Joint FAO/IAEA Centre
Nuclear Techniques in Food and Agriculture

International Symposium on

Sustainable Animal Production and Health

Current status and way forward

28 June to 2 July 2021
Vienna, Austria

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Application of improved technologies for sustainable livestock productivity: The way forward

Preface

The increased demand from consumers for milk, meat and other livestock products and animals has forced the producers to intensify their systems of production. The manifestation and its consequences are particularly relevant in the context of developing countries. This reality has brought together several challenges. On one hand, is how to increase productivity without degrading natural resources, including sources of food and feed and livestock genetic diversity. On the other hand, is how to ensure that transboundary animal diseases and zoonoses are detected early, diagnosed, and brought under control. Increasing demand can only be met through the selection of animals that produce more meat and milk and show disease resistance and heat tolerance, the optimal utilization of local resources that simultaneously protects animal biodiversity and the environment, and the protection of animals and their caretakers from diseases.

The International Symposium on Sustainable Animal Production and Health – Current Status and Way Forward, organized by the Animal Production and Health Section of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, found its departing point in these challenges. Within the five days of discussions and debates, the Symposium comprised a panel discussion and eight thematic sessions: a) molecular tools for animal production and health, b) advances in vaccinology, c) emergency preparedness and response, d) zoonotic diseases, COVID-19 and ZODIAC, e) enhancing livestock's contribution to One Health and the Sustainable Development Goals, f) challenges for better livestock production in the developing world; g) advances in biotechnologies for improving livestock breeding and feeding, h) application of improved technologies for sustainable livestock productivity: the way forward.

The symposium, held virtually, was attended by more than 3000 participants and observers from more than 160 countries, as well as by representatives of international organizations including the International Atomic Energy Agency (IAEA), the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (WOAH). The more than 50 presentations were related to research and development actions for the sustainable improvement of animal production and health, emphasizing the role of nuclear technologies. These presentations were complemented by more than 145 synopses and posters from the participants, which were made available in a Book of Synopses.

This publication is a compilation of the contributions emanating from the symposium. It encompasses the three opening speeches of the IAEA Director-General, Mr Rafael Mariano Grossi, the FAO Director-General, Mr Qu Dongyu, and the WOAH Director-General, Ms Monique Eloit; and 47 papers from participants and speakers, which have been peer-reviewed by FAO and IAEA colleagues, independent outside experts and the Scientific Committee. The Book of Proceedings provides vital information and evidence on how nuclear and nuclear related techniques can contribute to the development of sustainable livestock production systems, as well as noting the constraints and opportunities for their use in developing countries. The book hopes to serve as guidance for scientists as well as government and institutional policy and decision makers.

Acknowledgements

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Opening statements

Rafael Mariano GROSSI
Director-General IAEA

We are all here to attend a magnificent scientific event which is bringing together nearly 3000 scientists from more than 160 countries. We were hoping to have an in-person meeting but, unfortunately, the worldwide sanitary restrictions due to the COVID-19 pandemic did not allow it. Nevertheless, modern technology in telecommunications is facilitating the international interchange of knowledge and the possibility for people to meet via the Internet.

The International Atomic Energy Agency is well known for its role as a nuclear watchdog to verify the correct and peaceful use of nuclear energy; in addition, one of our clear mandates is to promote the application of nuclear technology through research and development in IAEA Member States, especially, in health, food and agriculture, environment, water resources and industry. The IAEA is in a unique position in the UN system having a multi-disciplinary laboratory in Seibersdorf, just outside Vienna, where nearly 400 scientists work on issues related to food and agriculture, human health, environmental monitoring and assessment, as well as the use of nuclear analytical instrumentation. The Animal Production and Health Laboratory is part of the Agricultural and Biotechnology Laboratories in Seibersdorf. This laboratory has proven to be crucial and highly relevant in supporting research and development work in Member States on zoonotic and transboundary animal diseases as well as in DNA based technologies for efficient management of animal genetic resources.

The opening of this symposium on animal production and health coincides with an important day for the livestock sector, especially for Africa. Ten years ago, 28 June 2011, the world celebrated the official eradication of rinderpest, a deadly disease that killed millions of cattle and wild animals and caused widespread starvation, mainly in Asia and Africa. The IAEA organized a side-event during the 2011 IAEA General Conference to highlight the efforts made during the eradication campaign and to underline what freedom from rinderpest meant to the world and especially to Africa. Several international organizations and donors contributed enormously to the control and eventual eradication of the disease. The Joint FAO/IAEA Centre and the IAEA Technical Cooperation Programme played a small but critical role providing technical expertise, training and support in the adaptation, validation and use of ELISA technologies for the monitoring of the rinderpest eradication campaigns in Member States.

Considering that smallpox was the first and only human disease globally eradicated and rinderpest was the second globally eradicated disease (first animal disease), much work is needed to mitigate transboundary animal and zoonotic disease incursions. Since early last year the world has faced another daunting threat, the COVID-19 pandemic, a disease that the world was not prepared for. The IAEA, based on the expertise gained when dealing with the rinderpest eradication as well as with other major outbreaks such as Ebola, Zika and avian influenza, was able to immediately respond and implement the largest technical cooperation initiative since its foundation. Nearly 26 million euros were kindly contributed by several Member States to fight the pandemic in nearly 120 other Member States. The Joint FAO/IAEA Centre, in coordination with the IAEA Technical Cooperation Department, was able to provide equipment, diagnostic kits, primers, and personal protective equipment to establish and implement molecular techniques to rapidly detect SARS-CoV-2. This successful initiative, and IAEA experience in assisting countries in the use of nuclear and nuclear-derived techniques for the rapid detection of pathogens that cause transboundary animal and zoonotic diseases, allowed for the design and initiation of a major project called ZODIAC (Zoonotic Disease Integrated Action). ZODIAC is operating under a holistic approach that ranges from identifying, monitoring, tracing, and early detection of zoonotic disease pathogens at the animal-human interface, to participation in global intervention and response to potential outbreaks. So far, nearly 150 IAEA Member States have joined the initiative, which aims to provide the world and specifically diagnostic laboratories with the knowledge and tools to identify and control major outbreaks before they can spread and destroy innocent lives.

This symposium is covering significant areas dealing with animal nutrition, reproduction, and breeding; as well as zoonotic and transboundary animal diseases, including COVID-19, to identify areas for more in-depth research and for ensuring food security and livelihoods.

QU Dongyu

Director-General FAO

I am pleased to be here today to open this international symposium which has attracted an impressive 2,000 participants from all over the world. These times have been unprecedented for all of us. The COVID-19 pandemic exposed the fragility of current animal production and health systems. A sustainable animal production and health system not only provides us with healthy and nutritious food, but also protects us from zoonotic diseases.

As many of you may know, FAO and IAEA have worked together since 1964 when we created the Joint FAO/IAEA Division to contribute to sustainable food security and safety through nuclear techniques and biotechnology. This successful partnership was enhanced and strengthened in February this year with the establishment of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

What is special about the Joint FAO/IAEA Centre is its dedicated multi-disciplinary laboratory that is unique in the UN system. The laboratory provides technical guidance, individual and group training, expertise and technical backstopping to our member countries. More importantly, it facilitates research to develop, adapt and validate nuclear and nuclear-derived technologies that can be applied in developing countries to improve animal production and control of diseases.

We work closely with the Animal Production and Health Laboratory in Seibersdorf, an WOAH Collaborating Centre for ELISA and molecular technologies in animal disease diagnosis. The Joint FAO/IAEA Centre is well known for the development, adaptation, and validation of diagnostic kits for the control and prevention of transboundary animal and zoonotic diseases. Millions of assay units were distributed to developing countries with laboratory support for its implementation, use and interpretation. This laboratory, and its VETLAB Network of veterinary diagnostic laboratories, was instrumental in the successful global eradication of rinderpest, which we will celebrate this afternoon.

The value of the Joint FAO/IAEA Centre laboratory technical support to member countries was also demonstrated during recent outbreaks of avian influenza since 2003, MERS in Near East (2016), Zika virus in Latin America (2016), Ebola in Africa (2014–2018) and the recent COVID-19 pandemic. The Joint Centre provided COVID-19 support to 128 Members with technical and expert guidance and services, equipment and reagents and consumables.

In the field of animal reproduction and genetics, the Joint FAO/IAEA Centre promotes the development and application of the novel and innovative techniques especially in developing countries.

FAO, IAEA and WOAH have a long history of working together on the prevention, control, and eradication of zoonotic and transboundary animal diseases. With different yet interrelated mandates, connected to One Health. One Health is essential to all, as it links human health, animal health and environmental health. The world is looking to us to produce synergies and provide leadership for the One Health approach to prevent future pandemics originating from animal sources. Protecting animal health under the One Health framework is at the core of our work.

The FAO Conference ten days ago endorsed our new Strategic Framework for the next decade. The Strategic Framework seeks to support 2030 Agenda through the transformation of more efficient, inclusive, resilient and sustainable food systems for better production, better nutrition, a better environment, and a better life (four betters), leaving no one behind. Sustainable animal production and health system are essential to attain these Four Betters.

This symposium is an excellent platform to discuss progress, but more importantly, to invest in the future. Let's join our efforts for One Health and to ensure safe and nutritional food for all.

Monique ELOIT

Director-General WOAH

It is my pleasure to take part in the opening session of the International Symposium on Sustainable Animal Production and Health.

Safeguarding animal health and welfare are at the core of the WOAH mission. Everyday our colleagues work to provide the WOAH members with information that is timely, transparent, and based on the latest scientific findings. We strive to assist our members on a daily basis, so they are capable of phasing endemic and emerging diseases threats with the best resources on the hand.

Even though the WOAH is better known for its standout on animal health and welfare we do not solely focus on animal disease control. The WOAH is making a long-standing investment in veterinary public health through data driven initiatives, monitoring and evaluation of its flagship programmes, and inclusion of sustainability at the core of all its projects.

In the global animal health, we are on demand. Working partnerships like the one with have with FAO and IAEA are crucial. Open communication allows us to find synergies which prove the work of each organization, and which benefit its member immensely. Open communication also avoids duplication of work or unfortunate overlapping at the time when our challenges are plentiful. We must work together for the global animal community with One Health at the core of its values.

The topics addressed in the present symposium are highly relevant to support sustainable animal production through the use of new technologies or adapting of existing techniques. Regardless the disease it is important to have efficient diagnostic tools, effective vaccines, but also surveillance and laboratory networks with experienced staff well trained with new technologies.

The IAEA programmes are essential in this regard and the Joint FAO/IAEA Centre even more. That is why I am please today to recognize the cooperation between our organizations, and I am looking forward to supporting further developments of this partnership to better continue to meet the new challenges facing our members.

The eradication of rinderpest, of which we are celebrating the 10th anniversary, is a perfect example of effectiveness of a well-built partnership.

At the time when the demand for animal products is intensifying in some parts of the world in order to feed a growing population the very principle of animal production is being strongly questioned in other regions. There is an urgent need to move forward with reflection and, above all, the operational implementation of plans on sustainability on agriculture, livestock, and aquatic production to reduce the impacts on natural ecosystems.

This conference offers the opportunity to further work on that and I therefore wishful a successful and fruitful event.

Executive summary

Session 1: Keynote Speakers and Panel Discussions

Five keynote addresses were presented during this panel discussion: Changes in food habits – Societal concerns regarding food of animal origin by Andrea Gavinelli; Genomic technology contributing to sustainable livestock development in China by Ren Wang; Feed industry contributions by Daniel Bercovici; Challenges and opportunities in mainstreaming health objectives in livestock investments by Franck Berthe; and SARS, MERS and COVID-19 by Norbert Nowotny.

The main points emerging from this session were:

a. Animal welfare in Europe has improved, but there remains a need for more enforceable rules;

b. Shortcomings of current legislations must be addressed to close gaps and create synergies with other policies;

c. Rules considering new scientific evidence and technological developments must be updated;

d. Industry, regulators, and other stakeholders must work together to contribute to the sustainable supply of safe and healthy food;

e. Public investment in animal agriculture and research must be increased, including for animal nutrition;

f. Regulatory frameworks must be adapted and strengthened to enable innovative feed solutions;

g. Investing in livestock and One Health will contribute to healthier people, a healthier planet, and a healthier economy;

h. Digital gene banks will be power tools in the future for taking full advantage of the power of modern genomic and phenomics technologies.

Session 2: Molecular Tools for Animal Production and Health

Five keynote addresses were presented: Disease diagnosis by Cameron Stewart; Sequencing and whole genome sequencing by Alessio Lorusso; Vaccine matching and quality control by Aldo Dekker; Genetic improvement of smallholder dairy cattle for sustainable diary systems: traditional and genomic approaches by John Gibson; and Genomic approaches to unravel the genetic basis of productive and adaptive traits in livestock by Paolo Ajmone Marsan. Additionally, 17 synopses were presented by scientists from 14 countries.

The main points emerging from this session were:

a. Highly potent vaccine can compensate for poor vaccine match;

b. The quality control of the vaccine and the vaccination campaign are essential for successful disease prevention through vaccination;

c. Information and communication technology (ICT) and genomic enabled programmes provide good platforms to build research and to test interventions, as the costs of data systems, farmer engagement and genomic testing are covered; the new information is automatically integrated; the results are reliable; and the farmers are actively engaged;

d. Targeted sequencing and whole genome sequencing can mitigate future pandemics, improve knowledge on viruses, and facilitate the surveillance of known and potential pathogens in wild animals;

e. Biomarkers, especially MicroRNAs, are essential for improved detection of infectious diseases as they complements existing diagnostic technologies.

Session 3: Advances in Vaccinology

Six keynote addresses were presented: Low-energy electron irradiation for the generation of inactivated vaccines by Sebastian Ulbert; Irradiated vaccines by Elena Gaidamakova; Irradiated Haemonchus contortus vaccine in field trials by Jayanthe Rajapakse; Novel approaches for bacterial vaccines (CCPP) by Jörg Jores; Development of mucosal vaccine against peste des petits ruminants virus for small ruminants by Muhammad Salah Ud Din Shah; and Mucosal immunity in large animals by Jean-Pierre Scheerlinck. Additionally, 11 synopses were presented by scientists from 11 countries.

The main points emerging from this session were:

a. New approaches have been developed to irradiate vaccines by the uncoupling of DNA/RNA damage from protein damage during γ-radiation, based on the Mn^{2+} antioxidant (MDP) complex;

b. Synthetic genomics is a fertile field and provides a potentially superior approach for the development of vaccines, as it allows the creation of cells with properties unobtainable by conventional molecular biology methods;

c. Irradiated larval vaccine has shown good results for control of *Haemonchus contortus*, as optimal antibody levels can be maintained with only 2 doses;

d. A mucosal vaccine against *peste des petits ruminants* has also shown good results, especially relative to sub-cutaneous application;

e. Intrapulmonary vaccination with ISCOMATRIX adjuvant is very efficient;

f. Intranasal vaccination tends to be inefficient in the absence of excipients;

g. Inactivation of pathogens via low energy electron irradiation (LEEI) preserves antigenic structures while also inducing potent immune responses.

Session 4: Emergency Preparedness and Response

Five keynote addresses were presented: Peste des petits ruminants by Uwe Müller-Doblies; Neglected or underreported animal health emergencies caused by zoonotic arboviruses in Africa by Martin Groschup; Adaptation of techniques into laboratory networks by Trevor Shoemaker; The role of national authorities by Abel Wade; and Monitoring emerging zoonotic disease threats and characterizing behavioral risk in high-risk communities in Senegal by Modou Moustapha Lo. Additionally, 25 synopses were presented by scientists from 16 countries.

The main points emerging from this session were:

a. Studying exotic viruses at their present locations as well as supporting developing countries in establishing functional public and veterinary health systems are critical activities for comprehensive control and prevention of transboundary animal diseases;

b. Another essential aspect is the capacity building of infectiology diagnostics, in terms of facilities and personnel;

c. The preparation and response to emergencies must be done under the framework of One Health;

d. Implementation and maintenance of bio-risk management systems in veterinary laboratories are critical to prevent infection of operators and spillover of pathogens into the environment;

e. Biosafety approaches should be taken in the field to ensure human safety; to prevent the spread of infectious diseases within and between epidemiological units; to protect assets; and to guarantee the safe collection of high-quality samples;

f. To safeguard a proper biosafety system, the risk assessment for different activities should be done, involving the farm staff, adapting the control to the local settings, and rehearsing the response procedures;

g. Laboratory networks, with the sharing of harmonized and adapted techniques, are an essential component for improving surveillance, detecting cases, diagnosing diseases, and providing responses;

h. National authorities and governments play the main role in enacting the legislation that responds to a country's needs, hence making essential the participation of disaster management experts in the process.

Session 5: Zoonotic Diseases, COVID-19 and ZODIAC

Five keynote addresses were presented: WOAH programme for preventing and controlling zoonotic diseases by Mariana Marrana; Laboratory response for the control of zoonotic outbreaks: the Ebola example by Jacqueline Weyer; SARS-CoV-2: its origin and the role of animals in the COVID-19 pandemic by Marion Koopmans; The role of wildlife in the emergence and spread of zoonotic diseases by Melinda Rostal; and ZODIAC project by Gerrit Viljoen. Additionally, 16 synopses were presented by scientists from 13 countries.

The main points emerging from this session were:

a. Early warning systems and surveillance systems are indispensable for the quick detection and response in face of zoonotic diseases under the umbrella of One Health;

b. 60 percent of the emerging infectious diseases (EIDs) come from animals, and 75 percent of those come from wildlife;

c. Wildlife farming, wildlife trade and direct transmission to people are key components that need to be considered for the emergence and spread of zoonotic diseases;

d. The Zoonotic Disease Integrated Action (ZODIAC) project launched by the IAEA aims to strengthen zoonotic diseases control through five pillars: i) Strengthening detection, diagnostic and monitoring capabilities in Member States; ii) Development of novel technologies for the detection and monitoring of zoonotic diseases; iii) Support to the decision-making tools through an IT platform; iv) Access to data on the impact of zoonotic diseases on human health; and v) Access to an IAEA Coordinated Response;

e. SARS-CoV-2 can infect many animal species, and, in some animals, evidence for efficient interhost transmission has been found;

f. Even though it is likely that the SARS-CoV-2 originated in Rhinolophus bats further surveys are needed.

Session 6: Enhancing Livestock's Contribution to One Health and the SDGs

Seven keynote addresses were presented: Antimicrobial Resistance – laboratory, surveillance and research in the FAO Action Plan (2021–2025) by Francesca Latronico; Emergency management centre for animal health by Frédéric Poudevigne; Preparing laboratory services for major emergencies -laboratory contingency plans and simulation exercises: what can be done even in a virtual world by Pam Hullinger; Preparing laboratory services for major emergencies / diagnostic reagent banks: example of Balkans by Kiril Krstevski; Connecting animal movements and other risks to optimise surveillance in West Africa by Baba Soumaré; The role of the livestock master plan in the development of the livestock sector by Clarisse Ingabire; and Health hazards in animal feed by Gijs Kleter. Additionally, 8 synopses were presented by scientists from 7 countries.

The main points emerging from this session were:

a. Laboratory contingency plans are necessary to respond successfully to an animal health emergency;

b. The EuFMD online simulation exercises provide the possibility for participants to communicate directly using an online software and generate documents that allow them to replicate real-life situations and perform necessary tasks and functions;

c. Livestock Master Plans give decision makers necessary evidence to make strategic choices and attract investments, reducing greenhouse gas emissions and positively impacting climate change;

d. The importance of animal feed safety for animal and consumer health is increasingly being recognized;

e. The need for internationally harmonized guidelines and best practices has been recognized;

f. Reagent banks can make significant contributions to the readiness for rapid and accurate diagnosis, and can play an important role in annual performance tests, identification of training needs, and improvement of laboratory networking and collaboration;

g. FAO highlights antimicrobial resistance surveillance and laboratories, while providing support for improving data generation and management and hosting a data platform, assessment tools and technical resources;

h. Effectively addressing agro-crime and agro-terrorism requires interagency collaboration of WOAH, FAO and INTERPOL;

i. Livestock mobility, driven by climate, seasons, environment and socioeconomic reasons, plays a critical role in sustainable livestock production in West Africa, but also increases the risk of occurrence and spread of animal diseases;

j. West Africa requires a regional risk assessment approach to understand the drivers of disease spread and to ensure safe and sustainable mobile livestock.

Session 7: Challenges for Better Livestock Production in the Developing World

Six keynote addresses were presented: Evolving livestock sector: innovative technologies and their application in the developing world by Jeroen Dijkman; Transformation strategies to improve the water productivity in agriculture in semi-arid regions by Simone Kraatz; Adopting reproductive biotechnologies for animal breeding: issues in developing countries by Joao Henrique Moreira Viana; Domestic animal biodiversity loss: potential risks for global food security by Mary Mbole-Kariuki; and Livestock resilience to emerging diseases and climate change: a game changer to building back better food systems post-COVID-19 by Mohammed Shamsuddin. Additionally, 41 synopses were presented by scientists from 27 countries.

The main points emerging from this session were:

a. The Sustainable Development Goals highlight the need to urgently improve the performance of production and consumption systems, including agri-food systems;

b. Research and new technology can play a key role in driving these transformations considering four key issues: i) adopting scaling and impact perspectives, ii) linking to multi-stakeholder alliances aligned to sustainability, iii) engaging society, and iv) safeguarding the balance among the social, environmental, and economic contributions;

c. Climate change and water scarcity underline the importance of agricultural transformation strategies;

d. Measures that can be changed in agricultural scenarios include technical (changing irrigation management, reducing loss due to evaporation) and land use (reduce or change cultivate area, change the wasy land is used) practices;

e. The strategies to adapt farming systems to water scarcity require the synergy of stakeholders, production, policy, organization, and technology;

f. Challenges for better livestock production in developing countries can be addressed by increasing cryoconservation of endangered populations/breeds, intensifying selection programmes, increasing advocacy and awareness, adopting existing policy instrument tools, and strengthening skills and capacities to conserve biodiversity;

g. Livestock production systems require a consolidation of the science – policy interface to integrate scientific, indigenous, and local knowledge to support decision-making processes;

h. Assisted Reproductive Technologies (ART) have the potential to improve productivity and sustainability of livestock production, especially in developing countries;

i. The feasibility of the use of in vitro fertilization for livestock production in a country depends on scale and demand for genetic gains;

j. A stronger animal health system and improved wildlife management is needed to prevent the next pandemic, as the frequencies of emerging infectious disease events has increased over time;

k. Digital market platforms, productive partnership modalities and improved genetics, among other measures, can solidify a sustainable food system;

l. Both investments for climate change mitigation and for climate adaptation are necessary to reduce the impacts of climate change on livestock keepers and to improve their resilience.

Session 8: Advances in Biotechnologies for Improving Livestock Breeding and Feeding

Four keynote addresses were presented: Genomic revolution in livestock: advances in animal breeding

technologies for increased livestock productivity and sustainability by Jennie Pryce; Understanding endometrial function for fertility, food and health by Niamh Forde; Artificial breeding in practice for sheep and goat: the Sardinian example for the developing world by Maria Dattena; and Measuring feed intake under grazing and browsing conditions: an achievable task? by Robert Mayes. Additionally, 14 synopses were presented by scientists from 12 countries.

The main points emerging from this session were:

a. Artificial insemination for sheep and goats is a promising technology, requiring however constant updating and investment to ensure sustainability and continual improvement;

b. There are several key considerations to improve fertility and health of livestock: i) conservation/divergence of conceptus-maternal molecular signaling mechanisms, ii) understanding the influence of environmental extremes, and iii) male and female differences in the interactions;

c. Estimation of feed intake under grazing and browsing conditions becomes more challenging as livestock systems try to improve the efficiency making the measurement of diet composition more important;

d. There is a need for new methodologies for remote monitoring of animal feeding behaviour under extensive conditions, including the use of multiple methodologies together;

e. Genetics is important to combat climate change, through reducing greenhouse gas emissions and improving heat tolerance;

f. Considering farmer and consumer preferences in breeding objectives allows for proper focusing on sustainability, animal welfare and environmental impact;

g. Stable isotope technologies can be used to optimize approaches for achieving the dietary requirements of livestock managed under extensive grassland production systems.

Session 9: Application of Improved Technologies for Sustainable Livestock Productivity: The Way Forward

Four keynote addresses were presented: European Union actions for a sustainable and innovative animal nutrition sector by Wolfgang Trunk; Application of nuclear and genomic technologies for improving livestock productivity in the developing world: challenges and opportunities by Raphael Mrode; Strengthening breeding stock: strategies for implementing advanced embryo production technologies by Joao Henrique Moreira Viana; and Precision livestock farming and beyond by Tami Brown-Brandl. Additionally, 14 synopses were presented by scientists from 11 countries.

The main points emerging from this session were:

a. Precision livestock farming, by utilizing real time data on individual animals, facilitates the decision-making process;

b. Research in precision livestock farming can generate new products, and lead to better animal management, better understanding of the engineering facilities and better genetic selection;

c. Recent developments have broadened the potential uses of embryo transfer (ET) in livestock production, especially for developing countries;

d. In vitro fertilization (IVF) can be successfully used by small-farmers, and can play a role in controlling inbreeding and reducing genetic erosion;

e. Genomics can provide quick wins for developing countries and offer opportunities for across country or regional collaboration;

f. Genotypic data can be used to model underling genetics for resilience traits;

g. Bundled genomic services in combination with reproductive technologies are needed to improve cost-efficiency and facilitate widespread application;

h. More sustainable livestock feed sources are necessary, these may include seaweed and other feeds from the oceans, by-products from the food and biofuel industries and the bioeconomy in general, processed animal proteins, and insect derived feed materials;

i. Innovation in more sustainable feed additives reduces the negative impact of livestock farming on the environment, benefit animal welfare/health, and provides economic benefits;

j. Focusing on animal nutrition requires modern feeding systems and sustainable feed labelling for informed consumer choice.

Special Session: Commemoration of the 10th Anniversary of Rinderpest Eradication and Maintaining Global Freedom

The symposium had a special session commemorating the 10th anniversary of rinderspest eradication. The opening ceremony included the participation of the Director-General of FAO, Qu Dongyu, the Director-General of IAEA, Rafael Mariano Grossi, and the Director-General of WOAH, Monique Eloit. The session continued with the presentations of Junaidu Maina, Samia Metwally and Mariana Marrana regarding the post-eradication challenges and achievements. The critical technical laboratory diagnostic role of the Joint FAO/IAEA Centre's laboratory was evident in all the presentations. It highlighted the fit-for-purpose serological platforms (esp.

ELISA) supported by the Animal Production and Health Laboratory of the Joint FAO/IAEA Centre. A panel discussion led by Keith Sumption and Mark Rweyemamu explored vaccines, vaccination and herd immunity; diagnostics, the eradication programme management, and the lessons learned.

The main points emerging from this session were:

a. FAO and WOAH have overseen the eradication of rinderpest with the establishment of the FAO-WOAH Rinderpest Secretariat and Joint Advisory Committee in 2012;

b. The FAO-WOAH Rinderpest Joint Advisory Committee provides technical advice on the application of rinderpest holding facilities, virus sequestration policy, research proposals, the international preparedness plan and ad hoc technical issues;

c. FAO and WOAH have carried out continuous activities for the prevention of reemergence of rinderpest: advocacy meetings, both regional and international; Global Rinderpest Action Plan; rinderpest awareness raising; global inventory; and continued education.

Advances in vaccinology

Mucosal vaccination in large animals

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Abstract

Induction of mucosal immune responses remains one of the holy grails of vaccinology. Indeed, by preventing pathogens from crossing the protective mucosal barrier it might be possible to tackle the pathogen early on, thereby preventing it from proliferating, causing damage and taking hold within the host. However, immune tolerance to vaccines delivered to mucosal sites makes this a difficult task. Herein, we review progress made in the understanding of what is required to successfully induce mucosal immunity in sheep. We specifically focus on intrapulmonary and nasal vaccination routes, as these are the most important ones for protection against respiratory diseases.

1. Introduction

Vaccines are one of the most effective ways of controlling pathogens in livestock and thus increasing farm animal productivity. Systemic immunization, practiced with most licensed vaccines, generally induces very poor protective responses at mucosal sites (Su *et al.*, 2016). Yet, the vast mucosal surfaces of the body covering the gastrointestinal, urogenital, and respiratory tracts serve as common portals of entry for most pathogens. Hence, mucosal vaccines could play a key role in protecting these key entry points from microbial invasion. If successful, mucosal vaccination also has an advantage over systemic immunization in that it controls infection when the pathogen is still present in relatively low numbers.

Mucosal vaccination can trigger both humoral and cell-mediated immune responses, not only at local mucosal sites but also systemically (Holmgren and Czerkinsky, 2005; Brandtzaeg, 2010). Indeed, mucosal immunization induces both long-term B and T memory cells (Brandtzaeg, 2007; Sheridan and Lefrançois, 2011); these cells are directed to home to the mucosal membranes by tissue-specific homing receptors. Mucosal tissue homing properties of B and T cells are acquired by virtue of their close interaction with specialised dendritic cells (DCs) that have migrated with exposed antigens from mucosal sites. This allows them to colonize the lamina propria of mucosal tissues after their egression from lymph nodes into the blood stream.

For example, intestinal DCs ($CD103^+$, $CD11c^+$, $MHCII^+$) produce retinoic acid, which increases the expression of chemokine ligand 9 (CCL9) and integrins $\{\alpha4\beta7\}$ on the surface of activated lymphocytes and the expression of the receptor of the integrin (i.e. MadCAM) is highly enriched on the high endothelial venules (HEVs) of the vasculature supplying the intestine, allowing the activated lymphocytes to colonize the mucosa after extravasation (Berlin *et al.*, 1993). This close interaction does not take place in the case of systemic immunization, resulting in a much-reduced induction of a mucosal immune response.

The induction of immunoglobulin A (IgA)-secreting plasma cells and IgA^+ B lymphocytes near the mucosal surface with secretory IgA (S-IgA) in mucosal secretions represents the main effector mechanism of the adaptive mucosal immune response and is one of the most important indicators of potency of a mucosal vaccine. In addition, generation of tissue resident lymphocytes also serves as an important protective mechanism, specially by virtue of cytotoxic T lymphocyte (CTL) responses.

Mucosal CTL responses have been prominently validated for their role in protective immunity in several animal models of infection employing respiratory and enteric viruses as well as intracellular parasites (Bender *et al.*, 1992; Franco and Greenberg, 1995; Buzoni-Gatel *et al.*, 1997).

The mucosa-associated lymphoid tissue (MALT) represents a highly compartmentalized immunological system and functions essentially independently of the systemic immune apparatus (Holmgren and Czerkinsky, 2005). It consists of a diffuse system of lymphoid tissue found in various submucosal membrane sites of the body, such as the gastrointestinal tract, nasopharynx, breast, lung, salivary glands, eye and skin. Compared with systemic lymphoid tissues, the MALT is populated by functionally distinct B cell, T cell and accessory cell subpopulations with cell-specific recirculation behaviours among mucosal sites (Holmgren and Czerkinsky, 2005). Upon adequate antigen presentation by antigen presenting cells (APCs), stimulated lymphocytes colonize additional mucosal sites and develop the so called "common mucosal immune system". While in mice this concept is well established, it is less clear in larger animals that all mucosal sites are indeed linked, rather some mucosal sites appear linked (e.g. the gut-mammary link), while others are less so or not at all (e.g. gut and respiratory tract). Thus, mucosal immunity could in certain cases, but not always, provide a protective immune response at the entry portal of pathogens by neutralizing the pathogens before they could enter the system, even if the vaccine is delivered to a different mucosal site.

Despite the advantages of mucosal immunity, immunising through mucosal routes to induce such a response remains a challenge (Levine, 2000). Immune tolerance prevents the induction of immune responses to the vast number of antigens that we ingest and inhale daily. Indeed, continuously mounting immune responses to common foods and inhaled particles would be detrimental to the host. In addition, the presence of proteases as well as other components of innate immune barriers, such as mucins and low physiological pH, also serve as important obstacles. Below, we review some of the principles that allow antigens to bypass these mechanisms and induce immune responses at mucosal sites particularly associated with the respiratory tract.

2. Pulmonary mucosal immunization

The lung is an attractive mucosal delivery site because it has a very large surface area and because it is possible to access different areas within this organ by adjusting the particle size for vaccine delivery. As an experimental model, in sheep, we used a fibre-optic bronchoscope as it allows for delivery of precise amounts of material to specific locations within the lung. This is important, as it is also possible to go back repetitively to the precise lobes to which the vaccine was delivered to collect samples. Repeat sampling is done by delivering saline via the sampling port of the bronchoscope and immediately sucking the saline back up with a syringe (Snibson *et al.*, 2005).

Using the system described above, in 2008, Wee *et al.* (2008) performed such an experiment in sheep to analyse the efficacy of pulmonary administration of an influenza vaccine and the impact of using the ISCOMATRIX® adjuvant on the consolidated systemic and local immune responses. ISCOMATRIX® is a particulate adjuvant consisting of *Quillaia saponaria* extract, cholesterol, and phospholipid, combining to form cage-like structures typically 40–50 nm in diameter. Pulmonary immunization with as little as 0.04 µg influenza antigen with the ISCOMATRIX® adjuvant was able to induce serum antibody responses at least equivalent to those induced by 15 µg unadjuvanted antigen delivered subcutaneously and, in addition, had superior pulmonary antibody levels (Wee *et al.*, 2008). The antibodies were also able to neutralize viral agglutination of red blood cells (hemagglutination inhibition), a standard test with a high correlation to protective immunity. The induced immune response was dependent on the presence of adjuvant and was also observed in the unvaccinated lobes, suggesting that it spread throughout the entire lung. This work showed that pulmonary vaccination could be an important route for induction of immunity to respiratory pathogens.

Amorij *et al.* (2007) compared the mucosal as well as systemic immune responses in mice using an inulin-stabilised spray-freeze dried formulation of the influenza subunit vaccine delivered to the lung via insufflation with an aqueous solution of the subunit vaccine delivered either by intra-muscular or via insufflation into the lung. The particle size of the dry powder aerosols was smaller than that of the aerosol droplets of the liquid subunit vaccine and thereby were expected to be able to penetrate deeper into the lungs. Results showed higher serum IgG titres and mucosal IgA titres in the dry powder group, confirming our observations using a different animal model, sheep.

Direct delivery of hepatitis B vaccine incorporated in polylactic-co-glycolic acid (PLGA) microparticles into the lungs of rats was more effective at inducing significantly higher antibody titres than is pulmonary delivery of plain antigen (Thomas, Gupta and Ahsan, 2010). Size, as well as the net charge of the microparticles was found to be important in determining the amplitude and duration of the induced immune response. Indeed, pulmonary immunization of Hepatitis B surface antigen (HBsAg) encapsulated in 5 µm PLGA microspheres elicited a significantly higher immune response as compared to 12 µm microspheres (Thomas, Gupta and Ahsan, 2010).

In another experiment by the same group, modification of PLGA microparticles with polyethylenimine or

stearylamine induced a greater immune response than hydrophobic nanoparticles, due to their positive charge. This could be due to greater uptake by the alveolar macrophages.

Taken together these results confirm our understanding of how mucosal immune responses are induced following lung vaccination. Indeed, proteins as well as killed vaccines generally induce poor immune responses following vaccination through the lung unless adjuvants are used. This is possibly due to degradation of antigen and/or inefficient uptake by APC. The use of microparticles in this context could increase the uptake of antigens by the Microfold (M) cells seated in the epithelium as well as the intra-epithelial extended dendrites of the DCs thereby promoting a greater immune response. In addition, adjuvants also induce production of local cytokine which might also play a role (Wee *et al.*, 2008).

3. Nasal immunization

Intranasal vaccination has several advantages over conventional intramuscular vaccination since it can potentially generate strong immune responses at key sites of pathogen exposure such as the lungs. Intranasal vaccination is known to induce strong local and systemic immune responses in the respiratory and genital tracts, the latter being demonstrated in humans and mice, but less well documented in other species. This directivity is because the B cells induced by nasal associated lymphoid tissue (NALT) express α4β1 integrin and C-C chemokine receptor type 10 (CCR10), and therefore tend to strongly migrate to the respiratory and genitourinary tracts, whose cells are highly expressing their ligands, VACM-1 and CCL28 (Kunkel *et al.*, 2003; Lazarus *et al.*, 2003; Johansen *et al.*, 2005). The NALT acts as the inductive site of the mucosal immune response when exposed to an antigen. The NALT is the organized mucosal immune system in the nasal mucosa, consisting of lymphoid tissue, B cells, T cells and APCs which are covered by a layer of epithelial cells containing M cells (Kiyono and Fukuyama, 2004).

Nasal tissues are amongst the most easily accessible mucosal sites and should therefore be seriously considered when developing a mucosal vaccine. However, experiments with nasal delivery in mice are often unreliable, as it is unclear whether the immune responses observed are induced within the nasal mucosa or by the delivered preparation reaching other mucosal sites such as the respiratory tract and the digestive tract. This is because the volumes of vaccine generally used are much larger than the nasal cavity of these animals (Scheerlinck *et al.*, 2008). In addition, the structure of the organised lymphoid structures associated with the nasal cavity are fundamentally different in mice compared to humans and livestock (Scheerlinck *et al.*, 2008).

Hence, we developed a sheep model in which immune responses in the nasal area could be measured in lymph draining from the head area, thereby excluding contributions from other parts of the body (Scheerlinck *et al.*, 2006). Using this methodology, we found that nasal delivery results in only extremely small amounts of antigen draining into the local lymph, with the majority being channelled away into the digestive tract through ciliary action at the base of the nasal cavity. Hence, there is a need to promote the uptake of antigen through the mucosal surface to ensure that enough antigen is available to the draining lymphatics.

4. Uptake through the mucosal surface by targeting the antigen to M cells

M cells actively recognize and transport particulate antigens such as viruses and bacteria across the epithelial layer by the process of phagocytosis. M cells structurally have less dense glycocalyx on their surface and morphologically have a basolateral pocket, allowing them to closely interact with B cells, T cells, macrophages and DCs, which are localized within the pocket (Neutra, Frey and Kraehenbuhl, 1996). These M cells are specialised in the transportation of antigen across mucosal surfaces and in transferring antigen to DCs, which in turn could bring it to the local lymph node where immune responses are induced.

Castelyn *et al.* (2013) compiled and reviewed the key markers of M cells comparing markers in MALT of humans, and domesticated and laboratory animals, including mouse, rat, rabbit, pig, cattle, horse and chicken. From this review Glycoprotein 2 (GP_2) appears as a promising candidate to target antigen to M cells to enhance antigen uptake and hence increase immune responses. Since GP_2 was not available for sheep, we cloned and expressed this protein to produce antibodies against the sheep GP_2. These antibodies could recognise GP_2 on the surface of M cells in palatine tonsils of sheep (Saxena, Diaz and Scheerlinck, 2019). Hence these antibodies might be used to increase effective transport of antigen into the sub-mucosal layer where the DCs reside, possibly leading to better immune responses to nasally-delivered vaccines.

In a significant recent development, Polylactic acid (PLA) and Polylactic acid-co-glycolic acid (PLGA) particles-based nanoparticles have been developed for M cell targeting specially by the oral route for a multitude of vaccines, including hepatitis B surface antigen (HBsAg), ovalbumin (OVA) and others (Roth-Walter *et al.*, 2005; Garinot *et al.*, 2007; Gupta *et al.*, 2007; Mishra *et al.*, 2011). However, most of the studies have been done in guinea pigs, a small laboratory animal, and suffer from the same drawbacks outlined above for mice, namely the spread of antigen to different sites following nasal delivery.

Chitosan is a linear amino polysaccharide obtained by partial deacetylation of chitin (polymer of *N*-acetyl D-glucosamine) (Singh *et al.*, 2018). Chitosan nanoparticles by their native mucoadhesive properties tend to reside longer in the mucosal surfaces, leading to better internalization of antigen through M cells and/or epithelial cells in the mucosal routes (Díaz *et al.*, 2016). In a guinea pig model against Foot-and-Mouth Disease Virus (FMDV), chitosan nanoparticle vaccines were shown to be effective at inducing potent mucosal responses. The chitosan used therein was of fungal origin and was characterized by a high degree of deacetylation, in addition to having a low molecular weight (Tajdini *et al.*, 2014). The degree of deacetylation of the particles directly impacts their physical properties including their molecular weight, solubility and biodegradability, antigen binding capacity and adjuvant activity. To further improve their immunogenicity, they could also be functionalized with M cell targeting ligands. Importantly, they have also proven to be effective as intranasal vaccine delivery systems for cattle and sheep (Günbeyaz *et al.*, 2010; Díaz *et al.*, 2016). Thus, use of chitosan nanoparticles *per se* or after functionalization with M cell specific targeting antibodies, may be a promising approach for nasal/oral vaccine delivery in large animals.

5. Conclusion and further experiments

Mucosal vaccination of large animals, and possibly also humans, through intra-pulmonary and intranasal routes is possible. Careful consideration needs to be given to choosing a suitable adjuvant and incorporating a mechanism to help ensure that the antigen reaches areas where mucosal immunity can be induced. In particular, intra-pulmonary vaccination with extremely low doses using ISCOMATRIX® demonstrated that this is a viable option under certain circumstances. Intra-nasal vaccination requires the antigen to be taken up into the locally draining lymph, and targeting M cells could increase this uptake. Further experiments will determine whether this is indeed the case, and most importantly whether this also increases the immune response induced via this route.

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Development of a mucosal vaccine formulation against peste des petits ruminants virus for small ruminants

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Abstract

Peste des petits ruminants (PPR) is a highly contagious viral disease affecting mainly small ruminants. The disease is caused by the PPR virus (PPRV) and is associated with high mortality and morbidity causing severe economic losses. The principal method for the control of PPR is subcutaneous (S.C)vaccination with a live attenuated vaccine like the PPRV/Nigeria/75/1 strain. Needle vaccination is costly as it requires professional staff to apply. Therefore, we developed an ocular vaccine formulation based on the PPRV/Nigeria/75/1 strain to allow the application by trained vaccinators. Different formulations of cryo-protectants and viral concentrations were tested in goats and the immune response was determined by ELISA and a virus neutralization test. The immune response of the vaccine administered by the ocular route was compared to a conventional subcutaneous route of vaccination using same viral concentration per animal. A dose of 100 ul ocular vaccine, having a viral concentration of 10^5 $TCID_{50}$/ animal with sucrose and lactalbumin as stabilizers was found to be optimum for a consistent immune response in tested animals. Animals vaccinated through the ocular route produced neutralizing antibodies with very similar patterns of antibody titres to the subcutaneous route over the 6 month follow up period. These promising results for the ocular application of a vaccine against PPRV in goats warrants further studies on the minimal protective dose of the vaccine virus and its formulation for heat stability to confirm if the ocular application could be used easily under field conditions for PPR eradication and control programmes in small ruminants.

Keywords

mucosal vaccine, immunity, peste des petits ruminants, SRMV, cell culture, VNT

1. Introduction

Peste des petits ruminants (PPR) is an acute, contagious and transboundary animal disease of small ruminants having high morbidity and mortality (Abu Elzein *et al.*, 1990; Radostits *et al.*, 2000; Brown *et al.*, 2011; Abubakar *et al.*, 2014). The disease is caused by PPR virus that is also known as small ruminants morbillivirus (SRMV) of family *Paramyxoviridae* (Barret, Banyard and Diallo, 2006; Amarasinghe *et al.*, 2017). The disease is characterized by the sudden onset of high fever, lesions on the tongue and dental pads, ocular and nasal discharge, diarrhoea and death (Diallo, 1988; Albayrak and Alkan, 2009; Ozmen *et al.*, 2009; Munir, Zohari and Berg, 2013). PPR has been reported in various countries of the world including Pakistan (Zahur *et al.*, 2011; Ullah *et al.*, 2015, Nizamani *et al.*, 2015; Ullah *et al.*, 2016), Afghanistan (Azizi, 2010; Banyard *et al.*, 2010), India (Chauhan *et al.*, 2011; Balamurugan *et al.*, 2012; Kumar *et al.*, 2013), Islamic Republic of Iran (Muthuchelvan *et al.*, 2014; Hemmatzadeh *et al.*, 2016), China (Wu *et al.*, 2016; Xia *et al.*, 2016), Bulgaria (Roberts and Gauntlett, 2018), Mongolia (Fine *et al.*, 2020) and others (Padhi and Ma, 2014; Parida

et al., 2016). It is considered a threat to small ruminant production in endemic areas (WOAH, 2021). Moreover, there is no antiviral treatment available and vaccination is the only option for prevention and control of the disease. Therefore, efforts have been made to develop effective vaccines for controlling the disease. Different types of PPR vaccines have been developed, such as conventional, recombinant and thermo-stable vaccines, for control and eradication (Diallo *et al.*, 2007, Chaudhary *et al.*, 2009; Baron, Parida and Oura, 2011; Riyesh *et al.*, 2011). The first attenuated vaccine was developed by using lineage I African isolate "Nigeria 75" (Diallo *et al.*, 1989b). Later on, three attenuated vaccines were developed by using lineage IV Indian strains (Diallo *et al.*, 1989a; Asim, Rashid and Noor, 2009; Saravanan *et al.*, 2010; Sen *et al.*, 2010). The need of a cold chain for transportation and poor thermo-stability were major hurdles in efficient use of these vaccines in countries with hot climates. (Siddique *et al.*, 1970; Worwall *et al.*, 2000). Thermo-stable live-attenuated vaccines have been developed to address this issue (Worrall *et al.*, 2000; Sarkar *et al.*, 2003; Intizar, Ahmad and Anjum, 2009; Riyesh *et. al.*, 2011; Mariner *et al.*, 2017). Currently, application of available vaccines requires subcutaneous injection, which requires trained personnel, relatively large volumes of vaccine, many consumables and extensive time and labour to vaccinate large herds of animals for proper disease control measures. Some researchers have tested the intra-nasal application of PPR vaccine and found that intra-nasal route is as effective as the subcutaneous route. However, in spite of this being a non-invasive and environmentally friendly method, these studies revealed that intra-nasal vaccine application is difficult in small ruminants. Researchers therefore suggested further long-term studies on intra-nasal vaccines in target species prior to its common use (Emikpe *et al.*, 2010; Ezeasor, Emikpe and Anosa, 2015; Jarikre *et. al.*, 2019; Mahapatra, Selvaraj and Parida, 2020). Alternatively, an ocular vaccine would greatly facilitate the mass vaccination campaigns for PPR eradication and induce mucosal immunity, reducing the risk of infection. Moreover, ocular vaccination would also be a non-invasive and environment friendly method because of its needle free application.

Thus, the aim of this study was to develop and investigate the efficacy of PPR vaccine for goats administered via an ocular route, and compare it to the subcutaneous application in goats.

2. Materials and methods

Viral strain and cells

The vaccine strain PPRV/Nigeria/75/1 was propagated in Vero cells (ATCC: CCL-81). The cells were cultured in Dulbeco's Minimum Essential Media (DMEM) supplemented with foetal bovine serum, streptomycin, penicillin and L-glutamine (Mariner *et al.*, 2017). The culture systems used for propagation of the virus were a stationary flask system and a roller bottle system.

Preparation of the vaccine

Vero cells having monolayer were infected with vaccinal strain (Nigeria 75/1) and the multiplicity of infection used was 0.005. The flasks/roller bottles were incubated at 37 °C in a carbon dioxide incubator. The cells were examined regularly for cytopathic effects (CPE). For this purpose, cell culture flasks were placed on the stage of an inverted microscope (Olympus) and observed under 10X and 40X objective lenses. When CPE were observed in 80–90 percent of the cells, the virus was harvested by freezing and thawing, titrated and stored at -80 °C.

Addition of stabilizers and lyophilization

Different cryo-protectants/stabilizers have been used to formulate different types of vaccines (Plowright *et al.*, 1970; Worrall *et al.*, 2000; Riyesh *et al.*, 2011). For this purpose, three different stabilizers were used: (i) Lact-albumin hydrolyste and sucrose (LS) (Merck); (ii) Trehalose di hydrate (TH) (TCI America); and (iii) Polyvinyl alcohol (PVA)(TCI America). Three different vaccines were formulated separately, having final concentrations of: (i) 2.5 percent Lact-albumin hydrolysate and 5 percent sucrose (LS); (ii) 17 percent trehalose di hydrate (TH); and (iii) 1.4 percent polyvinyl alcohol (PVA) to find the suitable stabilizer for ocular vaccine. Moreover, formulations of vaccines having different viral concentrations were also prepared to determine the lowest viral concentration needed to induce an immune response. Details about different formulations of vaccine are shown in Table 1, Table 2 and Table 3. The vaccine formulations were prepared and lyophilized according to standard protocols (Sarkar *et al.*, 2003).

Quality control and virus titration

Each vaccine formulation was reconstituted and its quality was tested prior to the start of animal trials. Quality control tests performed were sterility, safety and determination of $TCID_{50}$(Median Tissue Culture Infective Dose). Infective dose of the virus was quantified by determining the 50 percent tissue culture infective dose ($TCID_{50}$) in 96 well microtitre plates (BD Falcon). Briefly, ten-fold serial dilutions (10^{-1} to 10^{-7}) of viral samples were prepared in minimum essential media (MEM). Then, 100 ul of each viral dilution was placed in the 10 wells of the first seven rows (rows a–g). In addition, 100 ul of pure MEM was placed in the 10 wells the of last (h) row as a control (no virus added). Finally, 100 ul of Vero cells was added to each of the 10 wells of all rows (a–h) having 1 500 000 to 2 000 000 cells per ml of MEM. Micro-titre plates were incubated at 37 °C with 2 percent CO_2 and 45 percent humidity. Micro-titre plates were checked for cytopathic effects from Day 6 of incubation up to Day 12 and the end point titres were calculated (Reed and Muench, 1983).

Animal studies

A series of animal experiments was conducted to optimize the suitable formulation of the vaccine for ocular application and its immunogenicity was also compared with subcutaneous vaccination. During the study, all experimental goats were housed at the experimental farm of the Animal Sciences Division (ASD), Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The Institutional Animal Care and Use Committee (IACUC) of the Animal Sciences Division, NIAB, approved the study and all applicable international, national and institutional guidelines for the care and use of animals were followed.

The first animal trial was conducted to evaluate different cryo-protectants and stabilizers for their possible use with ocular PPR vaccine. Twenty sero-negative goats having 4 to 5 months of age were divided into four groups of five animals each. Three different PPR vaccines based on the Nigeria 75/1 viral strain were prepared with three different cryo-protectants (i.e. Sucrose with lactalbumin, trehalose and polyvinyl alcohol). The PPR vaccines were administrated as a primary dose at day 0. Animals from three of the groups were vaccinated through the ocular route of vaccination for one of the three vaccines, while a fourth group was treated as a negative control. Ocular vaccine was given at a rate 100 ul per animal (having 10^6 $TCID_{50}$ of virus) by placing 50 ul in each eye. Serum samples were collected at 10-day intervals continuously up to 2 months post-vaccination to determine the antibody titres of the experimental animals. The detailed experimental plan is shown in Table 1.

Table 1: Experimental design of the animal trial-1

Experimental group	No. of animals	Treatment	Dose/ animal	Composition of vaccine	$TCID_{50}$ of vaccine
1	5	Ocular vaccine	100 ul	Sucrose and lactalbumin	10^6 $TCID_{50}$/ animal
2	5	Ocular vaccine	100 ul	Trehalose	10^6 $TCID_{50}$/ animal
3	5	Ocular vaccine	100 ul	Poly vinyl alcohol	10^6 $TCID_{50}$/ animal
4	5	Negative Control	100 ul	Sterile phosphate-buffered saline	No virus

Source: Authors' own elaboration.

The second animal trial was conducted to determine the minimum viral titre of vaccine required for an effective immune response through mucosal application. Twenty sero-negative goats having 4 to 5 months of age were divided into four groups of five animals each. Three different PPR vaccines (Nigeria 75/1) were prepared having different virus titres (i.e. 10^6 $TCID_{50}$/animal, 10^5 $TCID_{50}$/animal and 10^4 $TCID_{50}$/animal) with sucrose and lactalbumin as stabilizers. The PPR vaccines were administrated as a primary dose at day 0. Three groups were vaccinated through an ocular route of vaccination while one group was treated as negative control. The ocular vaccine was given at 100 ul per animal in the form of eye drops to each experimental animal. Serum samples were collected at 10 days intervals continuously up to 2 months post vaccination to determine the antibody titres of experimental animals. The detailed experimental plan is shown in Table 2.

The third animal trial was conducted to compare the immune response of ocular and subcutaneous applications of vaccines, with or without booster doses. Experimental animals of each group were vaccinated with the vaccine having a viral titre of 10^4TCID_{50}/animal, with half the animals of each treatment receiving a booster dose using the same route of vaccination. Moreover, trehalose was used as a cryoprotectant/stabilizer in ocular formulation of vaccine and it was compared with subcutaneous formulation of a vaccine having sucrose and lactalbumin as the stabilizer. During this trial, immune response was monitored up to 6 months post vaccination. Twenty-five sero-negative goats having 4 to 5 months of age were divided into five groups of five animals each. Four groups were vaccinated through different routes of vaccination (subcutaneous and ocular routes) while the fifth group was treated as a negative control. PPR vaccine of strain Nigeria 75/1 was administrated as the primary dose at day 0 and booster doses were given at day 22. Groups 3 and 4

Table 2: Experimental design of the animal trial-2

Experimental group	No. of animals	Treatment	Dose/ animal	Composition of vaccine	$TCID_{50}$ of vaccine
1	5	Ocular vaccine	100 ul	Sucrose and lactalbumin	10^6 $TCID_{50}$/ animal
2	5	Ocular vaccine	100 ul	Sucrose and lactalbumin	10^5 $TCID_{50}$/ animal
3	5	Ocular vaccine	100 ul	Sucrose and lactalbumin	10^4 $TCID_{50}$/ animal
4	5	Negative control	100 ul	Sucrose and lactalbumin	No virus

Source: Authors' own elaboration.

were given only primary doses, while Groups 1 and 2 were given primary and booster doses of subcutaneous and ocular vaccines respectively. Subcutaneous vaccine was injected at 1ml per animal while ocular vaccine was given at 100 ul (50 ul in each eye) animal. Serum samples were collected repeatedly up to 6 months post vaccination to determine the antibody titres of the experimental animals. The detailed experimental plan is shown in Table 3.

Table 3: Experimental design of the animal trial-3

Experimental group	No. of animals	Treatment	Dose of vaccine	Composition of vaccine	$TCID_{50}$ of vaccine
1	5	Subcutaneous with booster	1 ml	Sucrose and lactalbumin	10^4 $TCID_{50}$/ Animal
2	5	Ocular with booster	100ul	Trehalose	10^4 $TCID_{50}$/ Animal
3	5	Subcutaneous single dose	1 ml	Sucrose and lactalbumin	10^4 $TCID_{50}$/ Animal
4	5	Ocular single dose	100ul	Trehalose	10^4 $TCID_{50}$/ Animal
5	5	Negative Control	1 ml	PBS	No virus

Source: Authors' own elaboration.

Serum antibody titres

Post-vaccination blood samples of experimental animals were collected up to 2 months for animal trials 1 and 2, and up to 6 months for animal trial 3. Sera were separated by centrifugation at 3000 rpm for 5 minutes and stored at -20 °C until further analysis. The serum samples of each experimental group were analysed via ELISA (Gür and Albayrak, 2010; Abubakar *et al.*, 2011) to determine the antibody titres against PPRV N-protein. We used a commercial ELISA kit according to the manufacturer's (ID Vet, France) guidelines. In the competitive ELISA assay, Percent inhibition values were calculated and values less than 50 were considered positive

Virus neutralization test

Virus neutralization tests (VNT) were performed to assess the comparative protection conferred by the vaccine through the different routes employed during animal trial 3. The VNT titre is the highest dilution of the serum that inhibited the cytopathic effect by 50 percent (Rossiter, Jessett and Taylor, 1985) and was expressed as $\log_{10}$ VN_{50}/ml. A neutralizing titre of greater than 10 is considered as positive. The VNT assay can detect the level of antibodies present in the serum capable of neutralizing the virus.

3. Results

Viral strain and cells

Typical CPE observed were cell rounding, cell ballooning, disruption of the monolayer and giant cell formation. The virus was harvested when 80–90 percent cells were showing CPE. The viral titre in the stationary culture flask was log $10^{5.5}$/ml and the titre in the roller bottle system was Log $10^{6.5}$/ml. After a few cycles of freezing and thawing, cryoprotectant was added and the virus was dispensed in the vials and lyophilized. Later, the vaccine was monitored for quality control measures, which included sterility, safety and determination of $TCID_{50}$ by Reed and Muench calculation method of 50 percent endpoint.

Animal studies

Animals were kept under observation during experimental periods. Body temperatures of animals were recorded regularly pre and post vaccination (data not shown) during animal trials. All animals were found to be normal, and no adverse effects were observed during the experiments.

The first animal trial revealed that all animals (5/5) of Group 1 showed immune responses. Experimental animals of Group 1 were vaccinated with vaccine having sucrose and lactalbumin (LS) as a cryoprotectant and 10^6 $TCID_{50}$ per dose of the virus. The same virus concentration with other formulations of cryoprotectants (i.e. trehalose and poly vinyl alcohol) did not perform well. Only one animal (1/5) of Group 2 showed an immune response; this group had trehalose as the cryoprotectant. None of the animals (0/5) responded in Group 3 with a formulation of poly vinyl alcohol. Therefore, sucrose and lactalbumin was found to be the suitable composition for cryoprotectant/stabilizer in ocular vaccine. Serum antibody titres of each group are shown in Figure 1.

During the second animal trial, experimental animals of each group were vaccinated with different viral titres (i.e. 10^6 $TCID_{50}$/animal, 10^5 $TCID_{50}$/animal and 10^4 $TCID_{50}$/animal having sucrose and lactalbumin as the cryoprotectant. All five experimental animals in each of Group1 and Group 2 showed immune response, while three experimental animals (3/5) of Group 3 showed immune response (Figure 2). Experimental animals of Group 1 and Group 2 received vaccines having viral concentrations of 10^6 $TCID_{50}$/animal and 10^5 $TCID_{50}$/animal, respectively. Therefore, the trial revealed that suitable minimum viral concentration for ocular vaccine was around 10^5 $TCID_{50}$/dose.

The third animal trial was conducted to evaluate the effect of booster doses on immune response up to 6 months post-vaccination. The ELISA serum antibody titres revealed a non-significant effect of the booster dose for both routes of vaccination. In the case of subcutaneous route, a

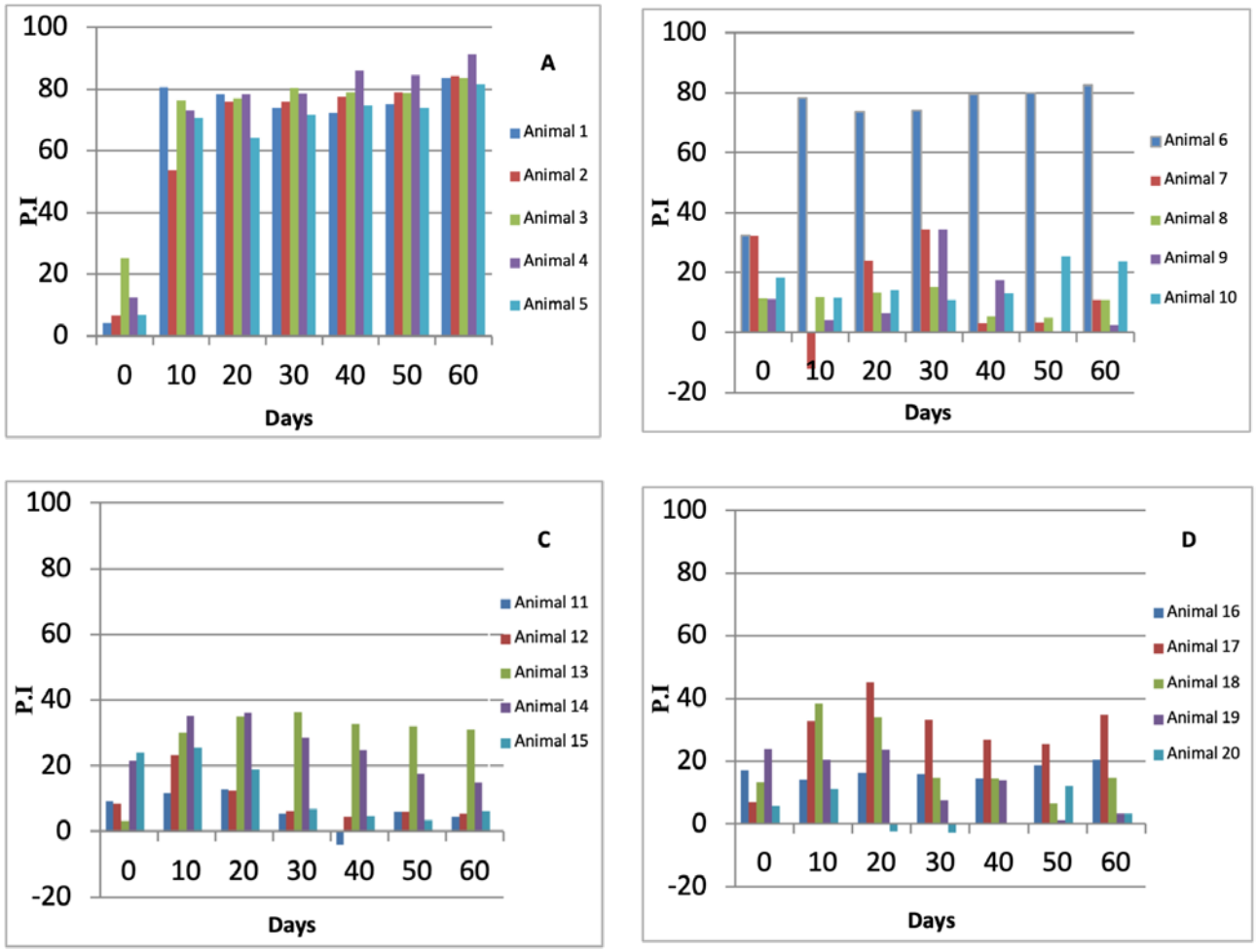
single dose of subcutaneous vaccine (Group 3) showed a better immune response (5/5) than Group 1 (4/5), which was treated with primary and booster doses. Similarly, in the case of the ocular vaccine, the booster dose did not make any difference. The single ocular vaccine (Group 4) showed immune response in 2 out of 5 animals, while 3 of the 5 experimental animals of Group 2, which were treated with primary and booster doses showed immune responses (Table 4, Figure 4). The serum antibody titres determined through ELISA and VNT are in agreement (Figure 3 and Figure 4).The serum VNT assay of experimental animals showed that ELISA-positive animals of each group had neutralizing antibodies within 10 days post vaccination. Moreover, both routes of vaccination (subcutaneous and ocular) induced the same pattern of immune response in positive animals up to six months post vaccination (Figure 3 and Figure 4). However, ocular vaccination with viral titre 10^4 $TCID_{50}$/dose induced immune response in 50 percent of the animals, while subcutaneous vaccine with same viral titre induced immune response in 100 percent animals (Figure 3, Table 4). The same results of ocular vaccine formulation were also found during animal trial 2 (Figure 2). So, it revealed that ocular and subcutaneous vaccine formulations having viral titres of10^5 $TCID_{50}$/dose are equally effective and ocular vaccine requires at least a 10^5 $TCID_{50}$/dose viral titre for optimum immune response in all vaccinated animals, whereas the animal experiment revealed that subcutaneous vaccine can properly induce the immune response even at a viral titre of 10^4 $TCID_{50}$/dose. Moreover, it was also found that a booster dose has no significant effect on immune response against either type of vaccines. The ELISA and VNT serum antibody titres of experimental animals are shown in Figure 3 and Figure 4 and Table 4.

Table 4: Comparison of virus neutralization tests (VNT) and ELISA for detection of antibodies against PPRV (NIG 75/1) pre-vaccination and post vaccination following initial and booster doses of subcutaneous and ocular PPR vaccines; each group comprised total five experimental animals

Days post vac.	Subcutaneous vaccine(1 × 10^4 $TCID_{50}$ /dose)				Ocular vaccine (1 × 10^4 $TCID_{50}$ /dose)			
	Vaccine + Booster (Group-1)		Single dose (Group-3)		Vaccine + Booster (Group-2)		Single dose (Group-4)	
	VNT*	ELISA*	VNT*	ELISA*	VNT*	ELISA*	VNT*	ELISA*
0	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
10	5/5	5/5	5/5	5/5	2/5	4/5	2/5	2/5
20	5/5	5/5	5/5	4/5	3/5	3/5	2/5	2/5
30	5/5	5/5	5/5	5/5	3/5	3/5	2/5	2/5
45	5/5	4/5	5/5	5/5	3/5	2/5	2/5	2/5
60	5/5	4/5	5/5	5/5	3/5	2/5	2/5	2/5
90	5/5	4/5	5/5	5/5	3/5	2/5	2/5	2/5
120	4/5	4/5	5/5	5/5	3/5	2/5	2/5	2/5
150	4/5	4/5	5/5	5/5	3/5	3/5	2/5	2/5
180	4/5	4/5	5/5	5/5	3/5	3/5	2/5	2/5

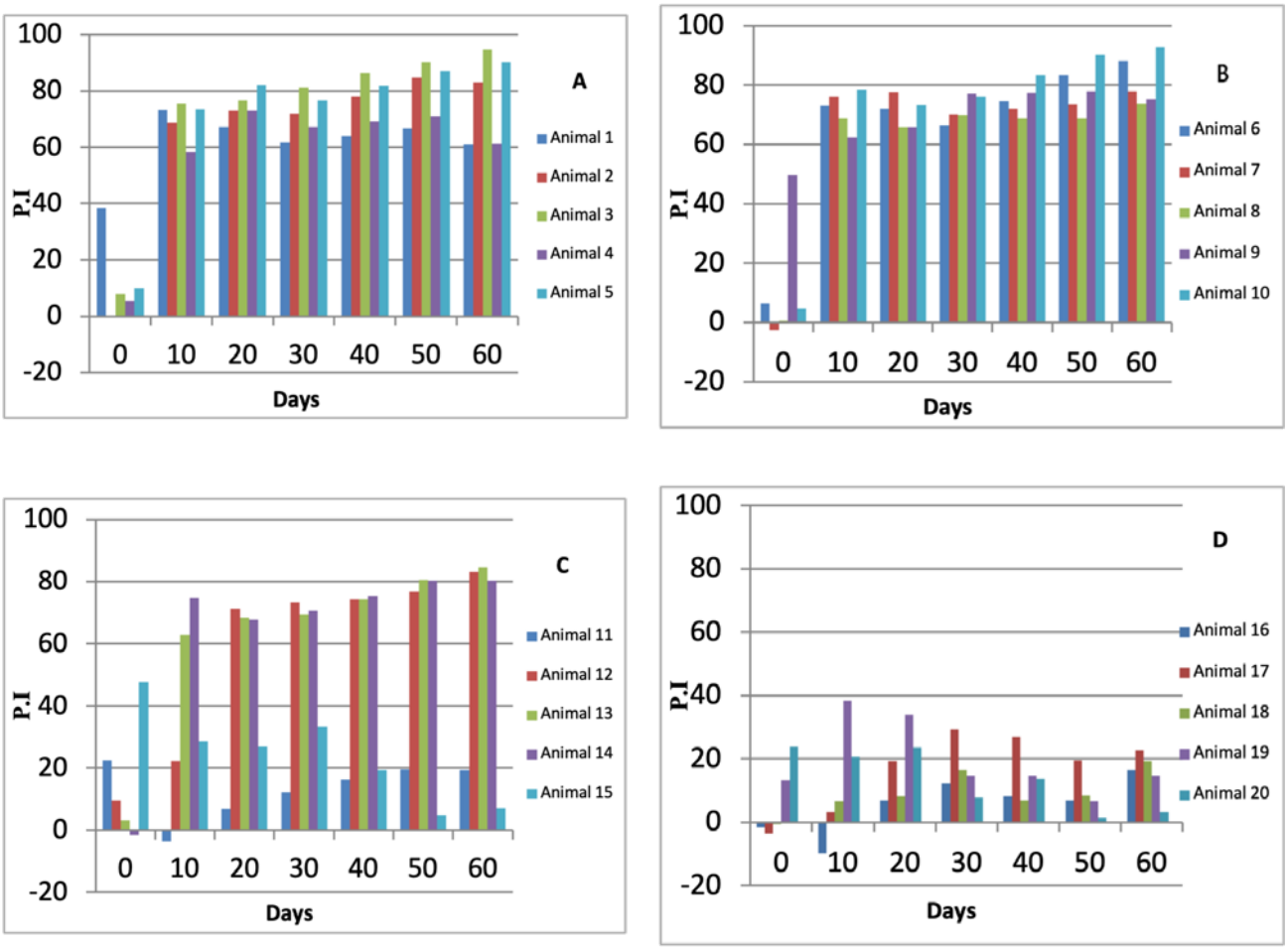
Source: Authors' own elaboration.

Note: * No. of VNT or ELISA positive animals/Total no. of animals in the group.



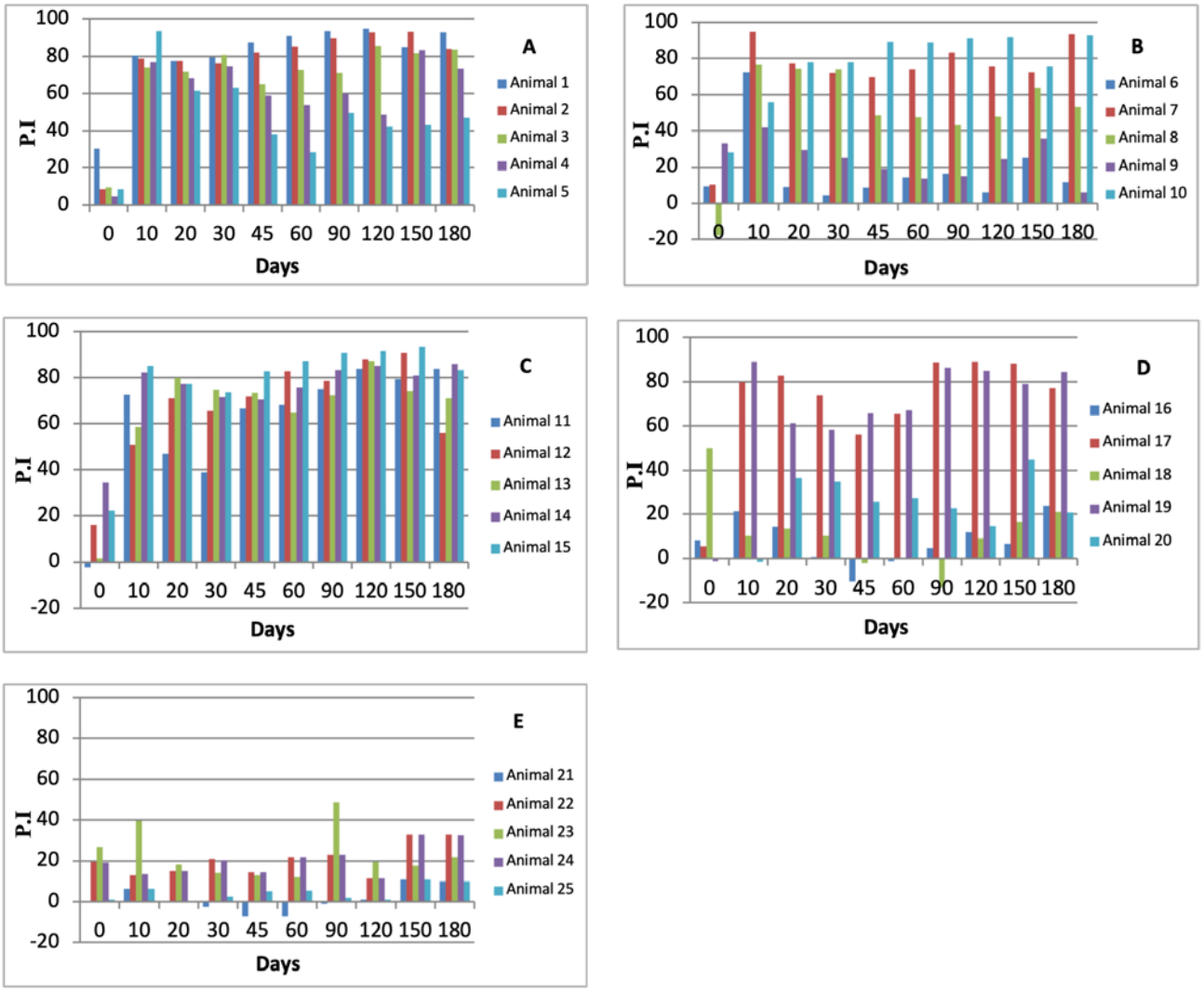
Source: Author's own elaboration.

Figure 1: ELISA serum anti-N antibody levels as percent inhibition (P.I) of experimental goats vaccinated with Nigeria 75/1 ocular vaccine having different stabilizers; (A) P.I ≥ 50 is considered to indicate positive immune response; (A) Group 1: Animals received a single ocular immunization with 10^6 $TCID_{50}$/ dose having sucrose and lactalbumin as the cryoprotectant, (B) Group 2: Animals received a single ocular immunization with a 10^6 $TCID_{50}$/ dose having trehalose as the cryoprotectant, (C) Group 3: Animals received a single ocular immunization with 10^6 $TCID_{50}$/ dose having polyvinyl alcohol as the cryoprotectant, (D) Group 4: Animals without immunization that served as a negative control



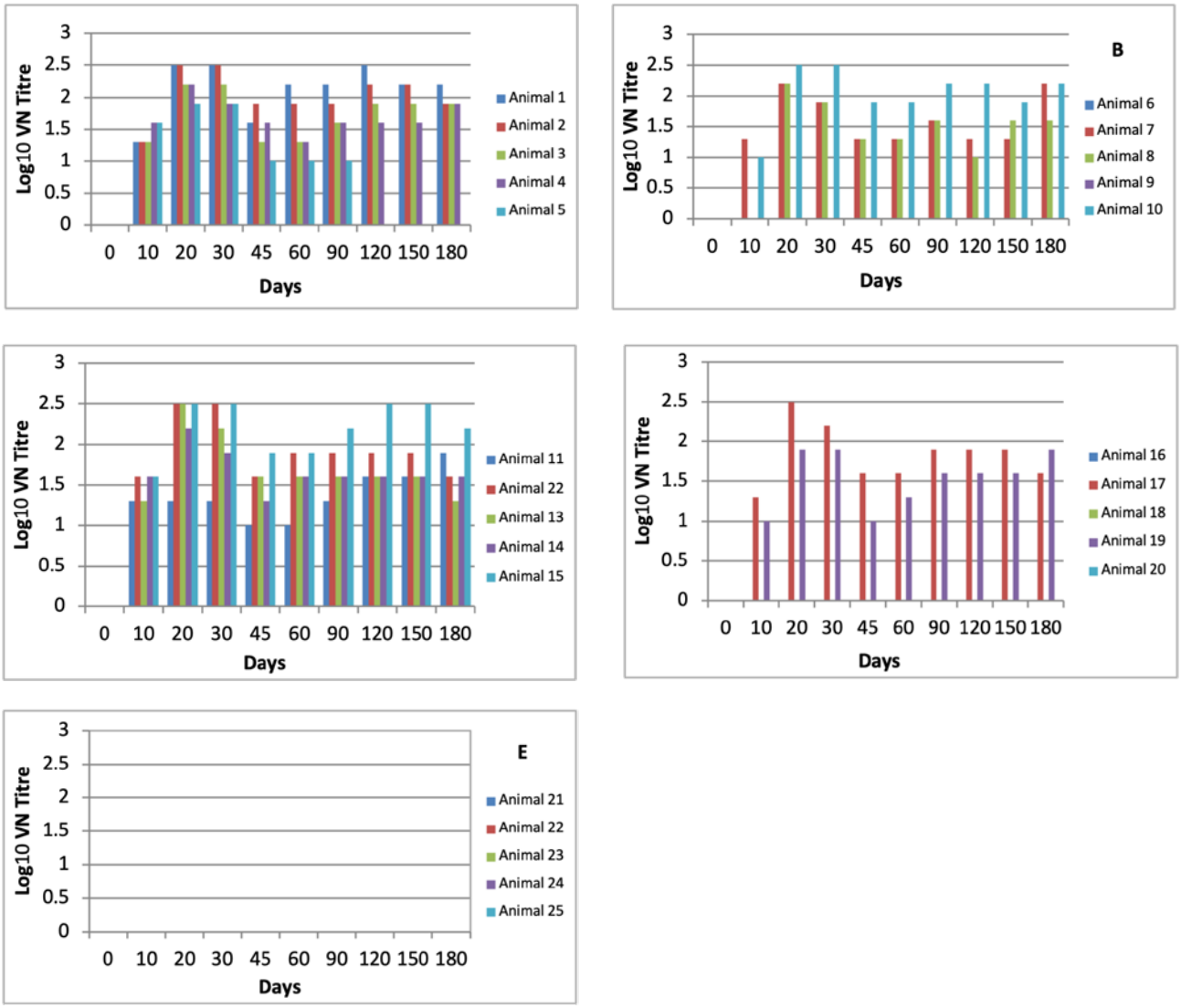
Source: Author's own elaboration.

Figure 2: ELISA serum anti-N antibody levels as percent inhibition (P.I) of experimental goats vaccinated with Nigeria 75/1 ocular vaccine having different viral titres (1 × 10^6 $TCID_{50}$/animal, 1 × 10^5 $TCID_{50}$/animal and 1 × 10^4 $TCID_{50}$/animal); A P.I ≥ 50 is considered as positive; (A) Group 1: Animals received a single ocular immunization with 1 x10^6 $TCID_{50}$/ dose having sucrose and lactalbumin as cryo-protectants, (B) Group 2: Animals received a single ocular immunization with log 10^5 $TCID_{50}$/ dose having sucrose and lactalbumin as cryo-protectants, (C) Group 3: Animals received a single ocular immunization with 1 × 10^4 $TCID_{50}$/ dose having sucrose and lactalbumin as cryoprotectants, (D) Group 4: Animals without immunization served as a negative control



Source: Author's own elaboration.

Figure 3: ELISA serum anti-N antibody levels as percent inhibition (P.I) of experimental goats vaccinated with Nigeria 75/1 1x 10^4 $TCID_{50}$/ dose; P.I ≥ 50 was considered as positive; (A) Group 1: Animals received subcutaneous immunization on days 0 and 22, (B) Group 2: Animals received ocular immunization on days 0 and 22, (C) Group 3: Animals received single subcutaneous immunization on day 0, (D) Group 4: Animals received single ocular immunization on day 0, (E) Group 5: Animals without immunization served as a negative control



Source: Author's own elaboration.

Figure 4: Virus neutralization test (VNT) serum antibody levels of experimental goats vaccinated with Nigeria 75/1 1x 10^4 $TCID_{50}$/ doses; VNT Titre ≥ 1 was considered as positive and expressed as log_{10} VN_{50}/ml; (A) Group 1: Animals received subcutaneous immunization on days 0 and 22, (B) Group 2: Animals received ocular immunization on days 0 and 22, (C) Group 3: Animals received a single subcutaneous immunization on day 0, (D) Group 4: Animals received a single ocular immunization on day 0, (E) Group 5: Animals without immunization served as a negative control

4. Discussion

PPR is an economically important disease of small ruminants. Prevention and control of the disease in endemic areas depends on vaccination. Moreover, PPR eradication campaigns have been started worldwide, and demand quick, easy and non-invasive administration routes of vaccination to cover large populations of susceptible animals. In the case of PPR, mucosal entry is the natural route of infection. The hypothesis of this study was that the use of natural route of infection for vaccination may trigger immune response through natural pathways and thus confer better protection than other routes of immunization.

During this study, efforts were made to develop a formulation for an ocular route of vaccination using the "Nigeria75/1" viral strain and its efficacy was tested in goats. Several ocular vaccine formulations were evaluated and compared with subcutaneous vaccine. ELISA and VNT assays were used to evaluate the immune status of experimental animals. Animals administered ocular and subcutaneous vaccines developed serum anti-N antibody and neutralizing antibody titres before the 10th day post vaccination. Moreover, experimental animals of both types of vaccines remained positive for serum anti-N antibodies and neutralizing antibodies throughout the study period (180 days). Ocular vaccine was also found to be safe, as no adverse reactions were observed in the vaccinated animals. In a recent study, anti-N antibodies and neutralizing antibodies were detected in goats following an intra-nasal vaccination of 2×10^5 $TCID_{50}$ (Mahapatra *et al.*, 2020), while an earlier study animals receiving 2×10^4 $TCID_{50}$ were negative for anti-N antibodies and neutralizing antibodies seven days post vaccine (Hodgson *et al.*, 2018).

During our study, we found that 1×10^5 $TCID_{50}$ ocular dose was sufficient to induce immune response (detectable anti-N antibodies and neutralizing antibodies) in 100 percent animals while 1×10^4 $TCID_{50}$ ocular doses induced immune response (detectable anti-N antibodies and neutralizing antibodies) in 50 percent of animals. Our findings are therefore in line with the previous reports. Moreover, we found that the serum antibody titres did not differ significantly between animals vaccinated either once or twice. Both ocular and subcutaneous routes of vaccination (having viral titre of 1×10^5 $TCID_{50}$/dose) were equally effective, as both routes induced almost similar patterns of humoral immune response.

We therefore conclude that a single dose of ocular vaccination having viral titre of 1×10^5 $TCID_{50}$/dose is sufficient to induce an optimum immune response. Given their similar efficacy and notable differences in ease of application, an ocular PPR vaccine may be the preferred option for vaccinating large populations of animals during mass vaccination campaigns for effective control of the disease. Moreover, ocular vaccination would have the added advantage of being a non-invasive method of vaccination as well as an environment friendly method because of its needle-free delivery.

5. Conclusions

We concluded that ocular and subcutaneous vaccine formulations (having viral titre of 1×10^5 $TCID_{50}$/dose) are equally effective in inducing immune response against PPR. The ocular application can be done by laymen, and because of its smaller volume, has additional advantages in production, cold chain storage and costs. Therefore, we suggested that a live attenuated ocular vaccine (Nigeria75/1) can be considered as a suitable candidate for mass vaccination campaigns and should be further evaluated in larger field trials. Further studies are required for evaluation of IgA levels expressed on the mucosa against vaccines with different routes of applications.

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Author contributions

Conceived and designed the experiments: HU, MH; Performed the experiments: MSS, MH; Analysed the data: MSS, MH, HU; Wrote the paper: HU, MH, MSS; Read and approved the manuscript: HU, MH, MSS.

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Compliance with ethical standards

Conflict of interest

The authors of this paper declare that they have no conflict of interest.

Ethical approval

The study presented in the manuscript did not involve any human subjects; it was an experiment on goats. The Institutional Animal Care and Use Committee (IACUC) of the Animal Sciences Division, NIAB approved the study, and all applicable international, national and institutional guidelines for the care and use of animals were followed.

Vaccine matching and quality control

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Abstract

Foot-and-Mouth Disease (FMD) is contagious disease of even-toed hoofed animals that has potentially high economic consequences. Control of the disease is mostly achieved by vaccination; therefore, testing efficacy of the vaccine is essential. The efficacy of a FMD vaccine depends on the potency of the vaccine as well the match between vaccine strain and outbreak strain. The different methods for potency evaluation as well as vaccine matching are discussed herein. Animal challenge related to vaccine evaluation is typically considered as the gold standard but is costly and raises concerns for animal welfare. To reduce the number of animals in challenge experiments, evaluation using *in vitro* methods is preferred. *In vitro* immunological tests have a good correlation with protection and can therefore be used to choose the vaccine based on potency and match between vaccine strain and outbreak strain. Moreover, these tests can also be used to evaluate the quality of the batch of vaccine that is acquired, as well as the quality of the vaccination campaign. Potent vaccines can often offer cross protection against strains that have a low vaccine match. Therefore, potency is often the most important characteristic in vaccine selection.

Keywords

Foot-and-Mouth Disease, vaccine, quality control, antigenicity

1. Introduction

Foot-and-Mouth Disease (FMD) is a contagious disease caused by FMD virus (FMDV) that can infect even-toed hoofed animals, which includes ruminants and pigs. There are seven serotypes (A, O, C, Asia1, SAT1, SAT2 and SAT3) of FMDV. On the farmer level, the economic consequences of an FMD infection can be enormous; especially in dairy cattle where loss of milk production is huge, but also, in fattening farms, food conversion drops. When cattle are used for draught power, the infection can result in the inability to plough the land, which can have huge consequences for crop production. On the country level, consequences are usually much greater, resulting from control measures such as culling and restriction of internal animal movement, in addition to subsequent suspension of export markets. Due to the direct economic consequences several continents have controlled FMDV infection and are now free of FMDV infection (Europe, North America, Australia and most countries in South America). This has led to trade barriers for countries that are not free of FMDV. To be able to bring milk and meat products on the international market and achieve a higher price for the products, freedom of FMDV is pursued in many regions.

For the control of FMDV, a management tool has been developed by the Food and Agriculture Organization of the United Nations (FAO) and the European Commission for the Control of Foot-and-Mouth Disease (EuFMD). This management tool is called "the Progressive Control Pathway for Foot-and-Mouth Disease" (PCP-FMD, www.fao.org/eufmd/global-situation/pcp-fmd/en). The PCP-FMD is a risk-based approach, and it does not prescribe the methods for control. In Europe, the risk for introduction of FMDV differs between countries in mainland Europe and the United Kingdom of Great Britain and Northern Ireland. The geographic isolation of the United Kingdom of Great Britain and Northern Ireland allowed the country to control FMDV by stamping out infected premises only, whereas countries in mainland Europe choose to control the infection by prophylactic vaccination. Both strategies worked; especially as the strategies were regionally coordinated by the EuFMD (Leforban and Gerbier, 2002).

As many endemic countries have direct neighbours and are not islands, FMDV control by prophylactic (and emergency) vaccination is usually the best tool. When using prophylactic vaccines, it is important to use efficacious vaccines. FMDV is genetically and antigenically variable. By definition there is not cross-protection between

serotypes, and even within a serotype the differences between strains can be huge. This phenomenon has serious consequences for the efficacy of FMD vaccine. Finally, in the field, the outcome of using a given vaccine depends on the strategy for its distribution and utilization. Vaccine campaigns can vary widely.

This paper will discuss the methods to select the best matching vaccine, as well as the methods to assess the quality of different vaccine batches and the quality of the vaccination campaign.

2. Foot-and-Mouth Disease vaccine matching

The best vaccine to control the circulating virus is a vaccine that induces the greatest immunity against the circulating strain. It is often assumed that the best immunity will be induced by vaccine strains that match very well with the circulating strain, but vaccine potency is also very important. Protection can be achieved by vaccines that induce a high level of immunity even if the vaccine strain has only a limited match to the circulating strain. The fact that the efficacy of the prophylactic or emergency vaccination is based on both the antigenic match between vaccine strain and circulating virus as well as the potency of the vaccine makes the selection of the best vaccine a difficult process. One could decide to perform an animal study with each vaccine batch using the circulating strains as a challenge virus, but this makes the vaccination policy more expensive and raises concerns about animal welfare. Moreover, in the case of emergency vaccination it is usually unwise to wait 4 to 6 weeks for the result of the challenge experiment. Therefore, *in vitro* methods are often the most logical approach.

Although *in vitro* methods for vaccine selection are preferred, the challenge tests are still the gold-standard for protection and, for this reason, we first highlight these standard tests:

PD_{50} study to determine vaccine potency

The WOAH manual (WOAH, 2021) describes two challenge experiments for testing FMD vaccine in cattle, (i) the protection against the generalized foot infection (PGP) test; and (ii) the 50 percent protective dose (PD_{50}) test. In the PGP test, cattle are vaccinated solely with a full dose and are recorded as either infected or not infected. Due to the dichotomous outcome of challenge, the power to quantify differences between vaccines is extremely low. In a PD_{50} test the potency of the vaccine is quantitatively assessed. In a PD_{50} experiment three or more groups of at least 5 cattle each are vaccinated with different fractional doses (e.g. full dose, 1/4 dose and a 1/16 dose). After 3 weeks (or longer if necessary) the vaccinated cattle together with 2 control cattle are infected in the tongue at 2 locations with 10 000 bovine ID_{50} (i.e. 50 percent bovine infectious dose) of virulent FMDV. Cattle that do not develop FMD lesions on their feet within 8 days after challenge are considered to be protected. Based on the dichotomous outcome (protected / non protected) the volume of vaccine that protects 50 percent of the cattle (PD_{50}) is calculated. The potency of the vaccine is expressed as PD_{50}/dose, which is the volume of a full dose divided by the volume that protects 50 percent of the cattle. A vaccine should contain at least 3 PD_{50} to be considered protective. To study the variability of potency tests, Goris *et al.* (2007) tested a FMD vaccine in 10 PD_{50} experiments and found 46/50 cattle protected at a full dose, 38/50 cattle protected at a 1/4 dose and 24/50 cattle protected at a 1/16 dose. The result shows that almost 50 percent of the cattle were already protected at a 1/16 dose. Using logistic regression, the overall outcome of the experiment was 15 PD_{50}/dose. The outcomes per individual experiment varied between 5 and 107 PD_{50}/dose. The variation in the 10 experiments was quite close to the outcomes when simulating the experiment in a Monte Carlo simulation (in 100 simulations the lower end of the 95 percent confidence interval was 3 PD_{50}/dose and the higher end was 66 PD_{50}/dose). The latter shows that almost all variation was random in the experiments and not systematic.

By using a PD_{50} experiment, the potency is quantified. When experiments are performed with homologous and heterologous challenges, the potency ratio can be calculated (Mintiu *et al.*, 1965):

$$Potency\ ratio = \frac{Heterologous\ potency}{Homologous\ potency}$$

The potency ratio is a measure of the antigenic similarity. In Europe, standard vaccines applied until 1992 had a potency of > 3 PD_{50}/dose, and with this, Europe was able to control FMDV. These vaccines normally contained strains that matched very well with the circulating strains in Europe at that time. This indicates that > 3 PD_{50}/dose against a circulating strain should be effective. According to Jamal *et al.* (2008) 3 PD_{50}/dose for oil vaccine correlates with approximately 71 percent protection (95 percent CI is 65 percent < protection < 76 percent). This implies that at least a 3 PD_{50} vaccine is needed to obtain more than 70 percent protection in the population. In an outbreak situation when only a few vaccine strains are available, one can compensate for the poor match by requesting vaccine with a higher homologous potency. For sufficient protection in the population (based on the criterion used in Europe when they were still practicing vaccination) the heterologous potency should be > 3 PD_{50}/dose against the outbreak strain. From this one can calculate the minimum homologous potency that is needed, namely 3/*Potency ratio.* For example, if the potency ratio is 0.2, one needs a homologous potency > 15 PD_{50}/dose.

The comparison between homologous potency (often estimated on the serological response) and heterologous potency has been studied for a number of vaccine strains (Brehm *et al.,* 2008; Nagendrakumar *et al.,* 2011; Fishbourne *et al.,* 2017; Dekker *et al.,* 2020b). The costs of these animal experiments are high and from an animal ethical point of view a non-invasive approach would be preferred. For this reason, serological assays using post-vaccination sera of cattle are mainly used to predict cross-protection.

Serological test for in vitro estimation of cross-protection

The WOAH describes a vaccine matching test by the virus neutralisation test (VNT), liquid phase blocking ELISA (LPBE) and the complement fixation test (WOAH, 2021). There is a paucity of data on comparing VNT, liquid phase blocking Elisa (LPBE) and complement fixation test in relation to protection, however. In many studies, a limited number of animals are used and tests are not compared statistically (Van Maanen and Terpstra, 1989). Analysis of unpublished data from South America comparing VNT titres tested in suspension, VNT titres tested on monolayers, LPBE and a monoclonal ELISA on 980 challenged cattle shows that the VNT titres tested on monolayers correlate better with true protection than the other tests, but all serological tests have a significant correlation with true protection. Although the WOAH suggests using VNT, laboratories in South America have opted for the LPBE because the results were more reproducible, both within a given laboratory as well as between laboratories.

When serological tests are used to determine the match between the outbreak virus and the vaccine virus, it is common to calculate the r_1-value

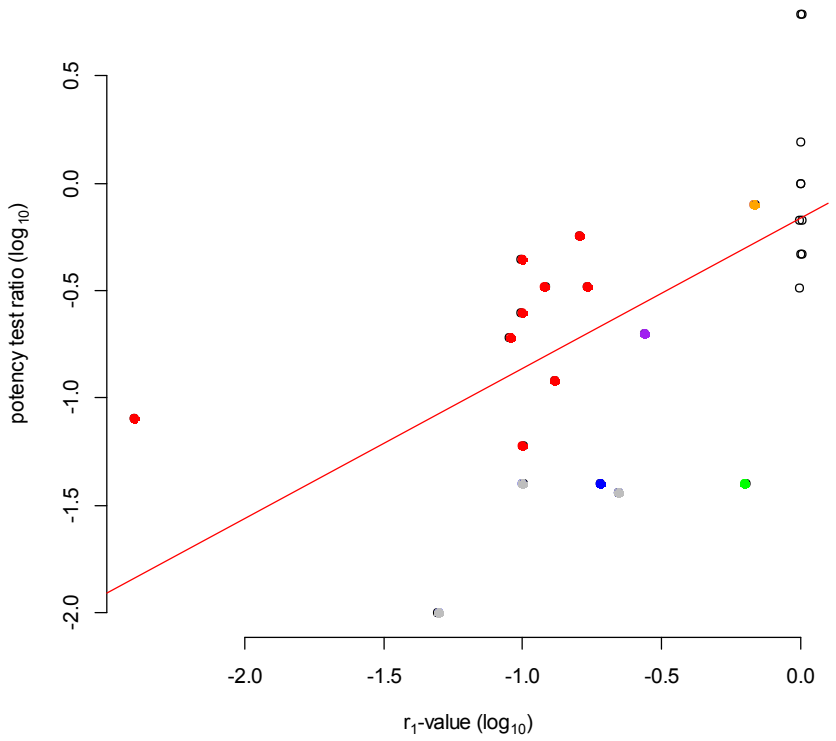
$$r_1\text{-}value = \frac{Arithmetic\ titre\ against\ the\ field\ strain}{Arithmetic\ titre\ against\ the\ vaccine\ strain}$$

When in a VNT an r_1-value of < 0.3 is found, the strains are considered significantly different. Due to the variation in VNTs, r_1-values above 0.3 do not indicate a significant difference between strains. In the LPBE the variation is smaller and an r_1-value < 0.4 would indicate a significant differences between strains. One should, however, realise that a statistically significant difference does not imply a practically relevant difference. In fact, previous studies have shown that vaccines made from strains with a low r_1-value can still sufficiently protect against other strains, at least when using vaccines with a high homologous potency (Brehm *et al.,* 2008).

As the formula of the r_1-value is very similar to the one calculating the potency ratio, it is logical to assume that they are related. Therefore, in my institute we compared the $\log_{10} r_1$-value and the $\log_{10}$ potency ratio determined in published and unpublished studies. We included the study in which 10 homologous O Manisa PD_{50} experiments were performed with the same vaccine (Goris *et al.,* 2007). By definition, this situation corresponds to an r_1-value of 1, which allowed the visualization of the variation in PD_{50} experiments (Figure 1). The variation in PD_{50} experiments is considerable, which explains why all observations in Figure 1 are spread around the estimate of the regression line. Although the regression is significant, the adjusted R-squared is only 0.35. This relatively low R-squared is probably caused by the variation in PD_{50} experiments and less by the variation in the serological tests. Variation in the serological tests can be reduced by repeating experiments. As serological responses (as well as cellular immune responses) correlate very well with protection, it is from a biological point of view logical to assume that the r_1-value is also a good predictor of the potency ratio. The fact that serological tests can be repeated more easily than challenge experiments supports the logic to perform *in vitro* experiments to estimate the vaccine match.

Although the VNT can readily be performed with every outbreak strain (if the strain grows in cell culture), the variation between tests is higher than when using ELISA. In our laboratory the standard deviation of the VNT is almost 0.3 $\log_{10}$ whereas we found a standard deviation of 0.15 in our swine vesicular disease LPBE. However, in a LPBE the antigen of an outbreak strain is not always recognised by the rabbit coating antibody or the guinea-pig tracing antibody. In a study where we compared the LPBE with the VNT and neutralisation index, we produced strain specific rabbit and guinea-pig sera for the LPBE. We observed that prediction of genetic differences (topotypes) was possible with the LPBE (significant receiver operating characteristic curve), but not with the VNT and the VI (virus isolation) (Tekleghiorghis *et al.,* 2014). It is not certain if the genetic differences between all topotypes are relevant for vaccine matching, but the result indicates that a more reproducible technique could have an advantage. The analysis of the previously discussed data from South America on 980 cattle tested with different tests indicated that the VNT is a better predictor of protection than ELISA (see above).

Novel approaches to improve the predictive ability ELISA tests would be beneficial. Mansilla *et al.* (2020) developed and tested one option whereby they purified the 146S (intact virus particles) and used these in the LPBE. This antigen purification improved the correlation with the VNT. The development of llama single domain antibodies that recognise 146S (Harmsen *et al.,* 2017; Li *et al.,* 2021) is a new option for the development of ELISAs that detect 146S antibody responses. The epitopes of these 146S specific llama single domain antibodies have now been mapped on the surface of the virus using CryoEM (Dong *et al.,* 2022). This knowledge will help to further develop better ELISA techniques that correlate with FMD vaccine induced protection.



Source: Author's own elaboration.

Figure 1: Relation between r_1-value and potency ratio in various animal studies: Open black circles 10 potency tests with the same vaccine (Goris *et al.,* 2007), in red 9 type A vaccines (Brehm *et al.,* 2008), in green (Nagendrakumar *et al.*, 2011), in blue (Dekker *et al.,* 2020b), in orange (Zheng *et al.,* 2015), in purple (Fishbourne *et al.,* 2017), in grey 3 non-published experiments (2 type SAT2 cross-protection studies and 1 type A study); the red line indicates the linear regression line

Genetic analysis for in vitro estimation of cross-protection

Nucleotide sequencing is very important for epidemiology; for example, geographically located genetic variants have been identified by using this technology. These geographical restricted genetic variants are called topotypes. Most topotypes circulate in a limited geographical area. For example, a study in South Africa found a topotype that was limited to only one wildlife conservation area, whereas in the other wildlife conservation areas other genetic variants were seen (Bastos *et al.,* 2001). These topotypes imply that the propensity of transmission of FMDV is limited in extensively reared livestock, although transmission in intensively kept groups can be rapid (Orsel *et al.,* 2009; Dekker *et al.,* 2020a). Spill over of FMDV from a region with a specific topotype into other regions, especially regions free of FMDV, can have dramatic effects, such as was seen for the 2001 outbreak in the United Kingdom of Great Britain and Northern Ireland (Gibbens and Wilesmith, 2002). In such cases, nucleotide sequencing gives information on the source of the outbreak virus. The source of the United Kingdom of Great Britain and Northern Ireland outbreak was probably illegally imported meat from Asia. In this case the source was not precise, but on a smaller scale, full-length sequencing can also be used to analyse the spread of FMDV within a country (Cottam *et al.,* 2006).

It is logical to assume that nucleotide sequencing can also predict vaccine matching. However, the prediction of antigenicity is limited when using nucleotide sequence as many nucleotide differences (often in the third base of a triplet) do not result in a different amino acid sequence. Reeve *et al.* (2010) showed that substitutions in surface-exposed structural proteins correlate to loss of cross-reactivity in VNT. This result suggests that the amino acid sequence together with structural information can be used to predict cross-reactivity in VNT, but data correlating these factors with cross-protection are not available.

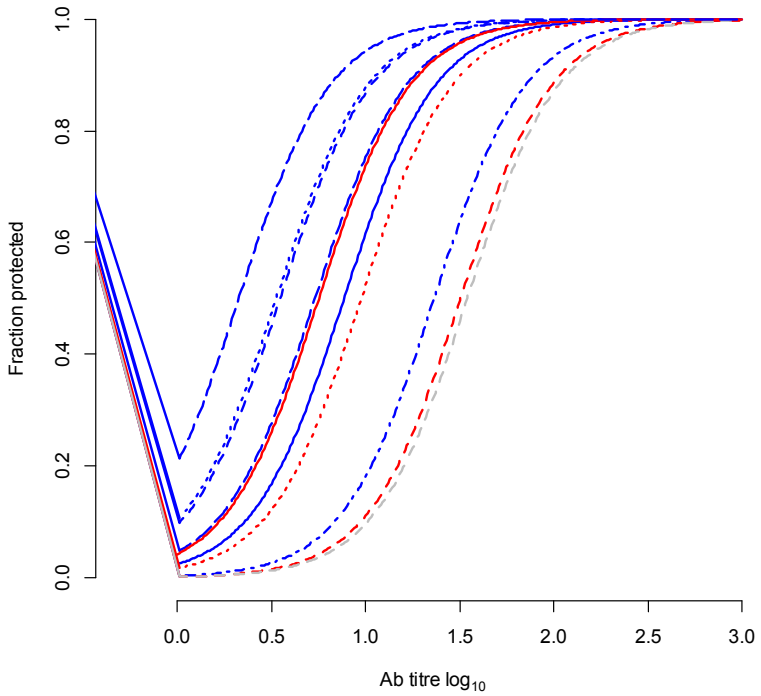
Conclusion – vaccine matching tests

From the reviewed literature, although *in vivo* cross-protection tests are the gold standard, the variation in these tests is considerable. *In vivo* serological tests correlate very well with protection and offer the opportunity to replace *in vivo* cross-protection tests, although for each strain it is essential to validate the relationship between homologous immune responses and protection. As sequencing becomes easier it will provide the first indications of which vaccine strain (topotype) is most likely the right choice; combining results of additional tests will help to guide the choice further. A very important point, though, is the result that high potency vaccines can induce cross-protection even if the vaccine match tests indicate a poor match. So, for quality control in the field, validating the potency of the vaccine is essential.

3. Foot-and-Mouth Disease vaccine quality control

FMD vaccine quality control according to good manufacturing practice (GMP) or other standards is performed by the vaccine producer; the producer shall follow Pharmacopoeia guidelines relevant for the country where the FMD vaccine is produced. If Pharmacopeia guidelines are absent, the WOAH manual (WOAH, 2021) can be used by the producer. The WOAH considers a product as an FMD vaccine when at least 3 PD_{50}/dose or 75 percent protection against homologous challenge is shown. When acquiring a vaccine, it is therefore important to evaluate the dossier to ensure that the criteria for efficacy and safety of the product are met. More information is given in the post-vaccination monitoring guideline (FAO and WOAH, 2016).

Although reputable producers do their best to produce good quality vaccine, not every batch of vaccine will be exactly the same, and during transport high temperatures can reduce the efficacy of the vaccine, it is therefore essential to evaluate each vaccine batch in a small number of naïve animals. Risks for degradation of the vaccine are not limited to the time of transport, they also occur during the vaccination campaign. Quality control of the vaccination campaign is thus also necessary. WOAH and FAO have therefore published post-vaccination monitoring guidelines (FAO and WOAH, 2016). The basis of the experiments described in these guidelines is the relation between antibody response and protection. Due to the fact that, in many studies, only one strain per serotype is studied, authors often suggest a different cut-off for each serotype. In the Netherlands, we have studied the relationships between the antibody titre from the VNT and protection for different strains from the same serotype (Figure 2). This analysis shows that the position of the relationship is different for each strain, but no significant differences in the slope of the curves were observed (Figure 2). The analyses yielding the data in Figure 2 were collected 21 days after vaccination; earlier studies have shown that the relationship is different (higher titre required to achieve protection) when tested 9 to 49 months after vaccination (Fish *et al.,* 1969).



Source: Author's own elaboration.

Figure 2: Relation between VNT titre (log_{10}) and protection for various serotype O (red), A (blue) and Asia1 strains tested in the Netherlands

When testing vaccines to evaluate quality as described in (paragraph 3.3) the post-vaccination monitoring guideline (FAO and WOAH, 2016), sera should be collected 21 days after vaccination and compared to sera from potency tests or international serological standards based on potency tests. The same guidance applies to sera collected in the field. As described in paragraph 3.4, sera should be collected at the time of vaccination and 21 days after vaccination, preferably from youngstock not previously vaccinated. The latter is important as the FMD vaccine challenge experiments are also performed in naïve cattle.

When vaccine is stored in a country and is approaching the end of its shelf life, a study as described in paragraph 3.3 of the guidelines (FAO and WOAH, 2016) can be

performed to see if the vaccine is still effective. However, new ELISAs have been developed that can evaluate *in vitro* the 146S content of a vaccine (Harmsen *et al.*, 2011; Harmsen *et al.*, 2015). During storage, the antigen disintegration of 146S to 12S will reduce the efficacy of the vaccine. In guinea-pigs, the immunogenicity of 12S was 70 times lower compared to 146S (Doel and Chong, 1982). These results show that the *in vitro* evaluation of vaccine by 146S specific detection of antigen can replace costly animal testing in some cases.

4. Conclusions

Highly potent vaccines can often protect against strains that have a low vaccine match. Therefore, not only vaccine matching is important, but also the assessment of the potency of the vaccine. For a country using vaccination, the quality of the vaccine should be assessed at the time the vaccine arrives in the country. But evaluation of the quality of the vaccination campaign is also essential, as poor vaccine efficacy in one region could become a source of new infections in other regions. When such problems are detected, administration of a booster in regions with a poor vaccine response will reduce the risk of new outbreaks.

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Protective efficacy of gamma irradiated avian influenza subtype H9N2 Iranian isolated antigen on broiler chicken

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Abstract

Immune response to gamma irradiated inactivated avian influenza virus (AIV) H9N2 Iranian isolate was evaluated in broiler chickens. Neutralizing antibodies and lymphocyte proliferation were measured by using the HI technique and a cell proliferation ELISA kit. The greatest protective response was shown in the group with gamma irradiated AIV H9N2 plus 20 percent trehalose. Irradiated AIV vaccine plus ISA70 and formalin vaccine plus ISA70 induced protective neutralizing antibody titers. Virus shedding was negative for five vaccinated groups 15 days post challenge, while being positive for the unvaccinated positive control group. Gamma irradiated AIV H9N2 virus is a good candidate for vaccine preparation and trehalose can be used as a stabilizer for vaccine formulation.

Keywords

avian influenza, gamma irradiation, inactivated vaccine, chicken, immune response, protection

1. Introduction

The most widespread influenza virus in poultry is low pathogenic avian influenza virus (LPAIV) H9N2 subtype (Nagy, Mettenleiter and Abdelwhab, 2017). The H9N2 subtype was reported in the industrial poultry populations of the Islamic Republic of Iran in 1998 (Tavakkoli, Asasi and Mohammadi, 2011). A variety of strategies can be used for prevention and control of avian influenza virus (AIV) infection in chickens, including increasing biosecurity, culling of infected animals, and vaccination (Astill, 2018). The conventional vaccine against AIV is inactivated by formalin, which damages the antigenic epitopes and might leave toxic residues in the vaccine (Furuya *et al.*, 2010; Motamedi-Sedeh *et al.*, 2015). The use of gamma (γ)-radiation for pathogen inactivation has been developed in the production of effective vaccines. The optimal irradiation dose for viral inactivation relies on the virus concentration, the size of viral genomes and the temperature when performing the irradiation (Müllbacher, Ada and Tha Hla, 1988). Gamma radiation targets the viral genome and only slightly affects viral structural proteins compared to formalin inactivation. Thus, a broader and stronger immunity should be induced in the host after the inoculation of the irradiated virus. Recent reports indicate that the immunogenic epitopes of gamma-irradiated AIV (γ-Flu) remain largely unchanged during the radiation treatment, resulting in a more potent induction of antibody- and cell-mediated immune

responses than with traditional vaccines (Alsharifi *et al.*, 2009; Furuya *et al.*, 2011; Russell, 2016). In this study, AIV H9N2 subtype was inactivated by gamma irradiation, and Montanide oil was used as an adjuvant. In addition, trehalose was used for vaccine formulation (Jain and Roy, 2009; Salehi *et al.*, 2018), as a protecting factor against stressors like irradiation, heat, cold, oxidation and desiccation. To test the efficacy of irradiated vaccine preparations, host trials including challenge studies were carried out to verify the level of protection for different formulations and applications (sub-cutaneous, intra nasal). Neutralizing antibody titres and lymphocyte proliferation assay were carried out to measure the humoral and cellular immune response.

2. Materials and methods

The locally isolated AIV H9N2 subtype strain A/Chicken/IRN/Ghazvin/2001 – a kind donation from the Razi Vaccine and Serum Research Institute of Iran – was used in this research. The titre of the virus was about $10^{8.5}$/ml EID_{50} before irradiation. The optimum dose of gamma radiation for complete inactivation of virus samples was 30 kGy according to the procedure described by Salehi *et al.* (2018). A Cobalt60 irradiator; the Gamma cell 220 (MDS Nordion, Ottawa, Canada) was used for irradiation. Formalin vaccine was prepared by using the Razi Vaccine and Serum Research Institute protocol (Amorij *et al.*, 2007). Montanide ISA70, as an adjuvant, was mixed with formalin inactivated or irradiated AIV H9N2 virus (70:30 V/V). In addition, 20 percent of trehalose (1 M) was added to some of the AIV subtype H9N2 virus stock before irradiation to protect against irradiation stress and to produce a more thermostable vaccine (Javan *et al.*, 2020).

Seventy broiler chicks (one day old, Ross 308) were purchased from the Hen & Chicken of Alborz Company (the Islamic Republic of Iran), individually weighed and randomly distributed into seven groups. The first group was injected subcutaneously (SC) with irradiated vaccine plus ISA70. The second group was injected SC with formalin vaccine plus ISA70. The third group was injected SC with irradiated vaccine plus trehalose. The fourth group was inoculated intra-nasally (IN) with irradiated vaccine plus trehalose, and the fifth group was inoculated IN with formalin vaccine. The final two groups served as controls. The sixth group was a negative control and was inoculated IN with sterile PBS. The seventh group was a positive control and was inoculated with 100 EID_{50} of AIV H9N2 as nose drops. The neutralizing antibody titration and stimulation index for lymphocyte proliferation assay were measured by using the **hemagglutination inhibition** (HI) technique and cell proliferation ELISA kit, BrdU (colorimetric) Roche Cat. No. 11647229001

The negative control and vaccinated groups were housed in two separate places. The positive control group was inoculated and housed in an isolated room. Vaccination was done at the first and 15th days post-hatch. The vaccinated and positive control groups were challenged with 100 EID_{50} of AIV H9N2 two weeks after the second vaccination intra-nasally (day 30). Blood samples were collected from the wing vein at 1, 15, 30 and 45 days post-vaccination and sera samples were stored at -70 °C for the neutralizing antibody response assay by hemagglutination inhibition (HI) test. The spleens of selected vaccinated chickens were removed aseptically at day 30 and 45 for the lymphocyte proliferation and interferon-γ (IFN-γ) assay. The IFN-γ assay was performed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and specific primers for IFN-γ. The relative results were calculated by the $2^{-\Delta\Delta CT}$ method to analyse the relative changes in gene expression (in relation to negative control group) from real-time quantitative PCR experiments (Livak and Schmittgen, 2001). Tracheal swab samples were collected at 1, 30, 32, 36, 38, 42, 45 days and placed in RNX-Plus solution (TRIzol) for total RNA extraction. Virus shedding was evaluated by qPCR, according to the protocol of Wee Theng Ong, *et al.* (2007) and a specific primer pair for H9 (Ong *et al.*, 2007). The sequence of the H9 gene of AIV subtype H9N2 strain, A/Chicken/IRN/Ghazvin/2001 was deposited in the NCBI database with accession number FJ794817.1 (1741 bp). H9 Forward and H9 Reverse primers and the FASTA format of the H9 gene (FJ794817.1) were aligned by MegAlign software. The alignment result showed the PCR product size was about 256 bp and it confirmed by agarose gel electrophoresis for RT-PCR product. RNA Mini Kit (Bio&Sell, Germany), EasyTM cDNA Synthesis kit (Parstious, Cat A101161, 50 reaction) and qPCR Mix EvaGreen kit (Bio&Sell, Germany) were used for RNA extraction from splenic lymphocytes and tracheal swab samples and real-time qRT-PCR tests. A serial dilution of cDNA for AIV H9N2 subtype was used to draw the standard curve.

3. Results

The experimental vaccination trial comparing different formulations of irradiated Flu vaccine versus the traditional formalin inactivated showed a rather positive outcome for the irradiated formulations, specifically with Trehalose intra-nasally, which had a 1 log higher antibody titre compared to the traditional formulation (Table 1). The lymphocyte proliferation measured on spleen samples incubated with irradiated virus and BrdU led to a significantly increased cell mediated immunity (CMI) response for the intranasal Trehalose formulation at day 30. This further increased after challenge as measured at day 45.

Table 1 shows the neutralizing antibody and stimulation index results for the seven experimental groups. Antibody titration in all vaccinated groups had significant increasing

two and four weeks after the second vaccination (30 and 45 days) compared to two weeks after the first vaccination (15 day). Also, it was the highest for the irradiated vaccine plus Trehalose ($p < 0.05$). The splenic lymphocyte proliferation assay showed significant increasing of stimulation index (SI) on all irradiated vaccine groups compared to other groups ($p < 0.05$).

The IFN-γ assay showed a significant increase between irradiated vaccine plus Trehalose (groups 4) and other groups ($p < 0.05$).

Virus shedding results are shown in Table 2. The Ct for negative samples is more than 20. Therefore, virus shedding for five vaccinated groups during 15 days after challenge was negative and it was positive for the positive control group only.

Table 1: Neutralizing antibody titres (by HI), Splenic Lymphocyte proliferation assay (by SI) and Fold Change of IFN-γ

No. group	Vaccine	Antibody titres				SI		IFN-γ; relative change $2^{-\Delta\Delta CT}$
		1d	15 d	30 d	45 d	30 d	45 d	30 d
	PreImmune	0^a						
1	Irradiated vaccine plus ISA70-SC		2.33 ± 0.57^b	4.33 ± 0.57^d	4 ± 1^c	1.14 ± 0.26^b	1.13 ± 0.47^b	$13,1\pm3.7$
2	Formalin vaccine plus ISA70-SC		2 ± 1^b	4.5 ± 1^d	4 ± 0.5^c	1.07 ± 0.53^a	1.08 ± 0.32^a	11.9 ± 6.177^c
3	Irradiated vaccine plus trehalose-SC		2.66 ± 0.57^b	4.75 ± 0.95^d	4.35 ± 0.95^d	1.20 ± 0.56^b	1.24 ± 0.36^b	16.4 ± 3.6
4	Irradiated vaccine plus trehalose-IN		3.33 ± 0.57^b	4.75 ± 0.57^d	4.5 ± 0.95^d	1.35 ± 0.41^c	1.52 ± 0.29^c	23.8 ± 5.5
5	Formalin vaccine IN		2.33 ± 0.95^b	3.75 ± 0.5^c	3.66 ± 0.81^c	0.95 ± 0.18^a	1.06 ± 0.23^a	5.12 ± 1.0
6	Negative control		0^a	0^a	0^a	0.89 ± 0.16^a	0.93 ± 0.25^a	1 ± 0^a
7	Positive control		Virus inoculation was done at 30 days and sampling at 4 and 15 days after challenge.					

Source: Authors' own elaboration.
Note: HI: Hemagglutination inhibition; SI: Stimulation Index; d: day; SC; subcutaneous; IN: Intranasal; IFN-γ: Interfron-gamma.

Table 2: QPCR results (Ct) of tracheal swap samples to evaluate virus shedding

No. group	Vaccine	1 d	30 d	32 d	36 d	38 d	42 d	45 d
	Pre-Immunization	UND						
1	Irradiated vaccine plus ISA70-SC		34.6	36.3	38.6	37.6	32.5	33.7
2	Formalin vaccine plus ISA70-SC		29.7	38.2	36.6	35.4	30.7	33.2
3	Irradiated vaccine plus trehalose-SC		34.0	34.0	37.3	39.5	35.0	33.0
4	Irradiated vaccine plus trehalose-IN		34.9	34.9	37.3	34.5	32.8	33.5
5	Formalin vaccine IN		35.2	36.3	UND	37.8	29.8	30.6
6	Negative control		UND			UND		UND
7	Positive control		UND		16.8			11.9

Source: Authors' own elaboration.
Note: UND: Undetermined; Cut off CT value: 20; SC; subcutaneous; IN: Intranasal.

4. Conclusions

30 kGy gamma irradiated AIV subtype H9N2 formulated with Montanide ISA70 or with Trehalose protects chicken against infection of corresponding influenza strains.

Montanide ISA70 can be used as an adjuvant for AIV vaccine formulation. Trehalose, when added as a radio-protectant, increases the protective efficacy for irradiated AIV subtype H9N2 vaccine. We observed higher antibody and greater lymphocyte proliferation response in groups treated with such vaccines compared to the traditional formalin inactivated vaccine. Interestingly the intra-nasal application of both vaccines led to a further increase of the cell mediated immunity after challenge. The irradiated Trehalose formulated Flu vaccine led to the strongest stimulation. Future trials will evaluate the long-term protection of these vaccine preparations in larger herds.

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Irradiated *Haemochus contortus* L3 larval vaccine in goats: Irradiation dose and protective immune responses

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Abstract

Haemochus contortus (*H. contortus*) is an economically important abomasal parasite of small ruminants. Due to the widespread resistance that has developed for all anthelmintic drug classes, vaccine production against *H. contortus* has been very much targeted during the last four decades. Despite many efforts and approaches taken by researchers worldwide, a cheap and highly effective vaccine against *H. contortus* is not yet available. The vaccine "Barbervax" has been shown to be effective in some species but has not been well tested in the goats. The goat industry in Sri Lanka is well developed and goat production is very common among small holder farmers, but it is affected by *H. contortus* infections, which cause severe economic losses. The current study was conducted to develop an effective γ-irradiated *H. contortus* L3 larval vaccine with low production costs and to evaluate the immune responses in local goats in Sri Lanka. An irradiation dose of 200 Gy in production of L3 larval vaccine was sufficient to develop lasting immune responses without causing the usual pathological changes that occur during natural *H. contortus* infection in goats. Two doses administered orally at days 0 and 35 led to development of humoral and cellular immune responses while the protective immune responses lasted for the entire 117-day experimental period. Thus, the irradiated *H. contortus* L3 larval vaccine developed in this study has high potential to be used effectively for controlling *H. contortus* infection of goats in the field.

1. Introduction

Haemochus contortus (*H. contortus*) is an abomasal parasite of small ruminants, causing production losses worldwide. In Sri Lanka, the goat industry is widespread and severe haemonchosis in goats is commonly observed in the dry zone region (Emery, Hunt and Le Jambre, 2016). Microclimate of the faeces, herbage and humidity play a major role in larval growth and prevalence. Usage of anthelmintic drugs in combination with grazing management and biological control are the main control measures adopted in the field.

The adult *H. contortus* nematodes live in the abomasum, consuming host blood. The severity of haemonchosis depends on the worm burden, age, breed, immune responses and the nutritional status of the animal. The adult *H. contortus* females are prolific egg layers, producing 10 000 eggs per day, and the eggs are passed out in the faeces where they hatch under favourable environmental conditions (Coyne and Smith, 1992). In the pasture,

development of first, second and third-stage (L1, L2 and L3) larvae take place. The L3 larvae are the infective larvae and, once ingested by the goats, develop into adult worms in the abomasum. During times of high humidity and temperatures, a greater number of L3 larvae tend to be found on tips of vegetation, increasing the possibility of transmission. The pathogenesis of haemonchosis is characterized by anaemia, with a decrease in packed cell volume (PCV) and haemoglobin concentration at the beginning of the third week post-infection. In severe infections, *H. contortus* may lead to loss of one fifth of red blood cell volume per day. Haemonchosis has three forms, acute, hyperacute and chronic, basically categorized depending on the number of infective larvae the host is exposed to and for how long (Naeem, Iqbal and Roohi, 2020).

Anthelmintic drugs can be grouped into several classes: benzimidazoles, probenzimidazoles, salicylamides and substituted phenols, imidazothiazoles, tetrahydropyrimidines, organophosphates, macrocyclic lactones and, more recently introduced, the amino-acetonitrile derivatives, namely cyclic octadepsipeptides, and spiroindoles. With the extensive usage and misuse of the above drugs for decades, *H. contortus* has acquired some level of resistance for all the anthelmintic drugs (Kotze and Prichard, 2016; Lamb *et al.,* 2017). Grazing management, using resistant breeds, and biological control strategies such as nematode trapping are currently incorporated together with the anthelmintic drugs to achieve the maximum effectiveness in controlling haemonchosis (Fernandes *et al.,* 2019).

Vaccination against haemonchosis has been under the discussion for decades. Recombinant proteins have shown some success and excretory-secretory products and adult membrane antigens such as H11, H-gal-GP, p26/23, Galα1Ga1NAc glycan epitopes and Hc-SL3 from *H. contortus* larvae have been extensively tested. Out of all the proteins evaluated, H11 has provided a 90 percent reduction in worm burden (Ehsan *et al.*, 2020). A commercial vaccine, Barbervax, which contains enriched antigens H-gal-GP and H11, has been launched and used (Kebeta *et al.,* 2021). However, as these antigens are "hidden" antigens, repeated vaccination is required to obtain high levels of protection (Nisbet *et al.,* 2016). Jacobs *et al.* (1999) reported significant reductions in egg count and worm burden by vaccination with a purified antigen (Hc-sL3) expressed on the surface of L3 larvae of *H. contortus*. But this study was only conducted in hair and wool sheep breeds. However, none of these vaccines have proven to be effective in controlling the *H. contortus* infection in goats.

For many years, attention has been given to the development of irradiated larval vaccines. Jarrett *et al.* (1959) evaluated the immunity produced by a single dose of *H. contortus* larvae irradiated with 10 000, 20 000, 40 000, 60 000 and 100 000 roentgens (87.7, 175.4, 350.8, 526.2, and 877 Gy) of X-rays. After the challenge, larvae irradiated with 40 000 and 60 000 roentgens stimulated good immunity in terms of reduction of worm burden. Later, Jarrett *et al.* (1961) tested a double-dose oral vaccine, with doses given 35 days apart, produced using 10 000 infective larvae of *H. contortus* irradiated with 40 000 roentgens (350.8 Gy) of X-rays. Sheep vaccinated in this way were able to withstand a challenge of 50 000 infective larvae, 29 days after the second dose of the vaccine (Jarrett *et al.,* 1961). No adult worms were found in the autopsy. Compared to the control group, vaccination provided 100 percent protection at day 16 after challenge. Challenge larvae did not develop beyond L4. In 1979, Ross, Duncan and Halliday (1979) reported reduction of *H. contortus* worm burden by 40 percent compared to the control, after challenge, by immunizing with two doses of 10 000 irradiated L3 larvae of *H. contortus* produced by exposure to a total radiation dose of 60 krad (600 Gy). Sivanathan, Duncan and Urquhart (1984) found that depending on the strain of the *H. contortus* larvae used, the irradiation dose may need adjustment to obtain similar immunogenicity.

The current study was conducted with the intension of developing an effective irradiated *H. contortus* L3 larval vaccine for goats.

2. Materials and methods

The experimental goats used in this study were all kept in a worm-free setting at the animal facility, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya. All animal experiments were approved by the animal ethics committee of the same faculty.

Vaccine development and irradiation dose testing

Vaccine development is dependent on the continuous availability of *H. contortus* larvae. To establish a source of larvae, the abomasa of goats infected by *H. contortus* were collected from the Kandy abattoir and they were transported in ice to the Division of Parasitology, Faculty of Veterinary Medicine and Animal Science. The abomasa were opened, and all female adult worms were collected under a white light. All female worms were crushed, and the eggs were collected into a cup containing distilled water. The collected eggs were mixed with dried sterile cow dung dust and hydrated with the distilled water until the mixture was moist and crumbly. The mixture was then transferred into wide mouth bottles, which were covered with a net. The bottles were incubated at room temperature for 14 days. The humidity of the mixture was maintained by daily spraying with distilled water. After 14 days, the L3 larvae were collected by the Baermann Technique. The larvae collected were stored in distilled water at 15 ℃ in cell culture flasks.

The collected larvae were then further developed and multiplied in live animals to obtain L3 larvae in quantities sufficient for preparation of the vaccine. Four, 3-month-old Saanan goat kids were brought to the animal facility and were treated with anthelmintics (Levamisole 0.1 g/10 kg body weight). The kids were allowed to get acclimated to the environment for two weeks. After two weeks, a sublethal dose of 10 000 L3 infective *H. contortus* larvae was administered orally. After 21 days of oral administration, the faecal matter was collected directly to a bag fixed via a harness. The faeces were collected, cultured and the L3 larvae were isolated as indicated above.

The L3 larvae were stored at 15 ℃ in cell culture flasks with distilled water supplement. The larval concentration was maintained at 10 000 larvae/mL. The larvae were transported to the Horticultural crop research and development institute, Gannoruwa, Kandy, on ice and irradiated using 25 kilo Gray of Cobolt-60 γ-irradiation. Three irradiation doses were used: 50, 100 and 200 Gy. One week following irradiation, the larvae were used as the vaccine.

To evaluate the effects of irradiation dose, 20 goat kids (3–6 months) were recruited and divided into four groups with five kids in each group. Group 1 was the unvaccinated and unchallenged control group, whereas Groups 2, 3 and 4 corresponded to each of the respective irradiation doses (i.e. 50, 100 and 200 Gy). Vaccinated animals were orally administered a sublethal dose of 10 000 L3 infective *H. contortus* larvae and received oral vaccinations at 0 and 42 days post-infection.

Faecal and blood samples were collected weekly from the rectum and jugular vein respectively. The faecal samples were evaluated for number of eggs per gram (EPG) while blood samples were used to monitor PCV, plasma protein concentration, white blood cell count, differential count and red blood cell (RBC) count using standard methods. The McMaster counting technique was used to evaluate the EPG. Briefly, 3 g of faeces was crushed and dissolved in 42 mL of saturated salt solution. The mixture was strained through cheesecloth to remove large faecal particles. The suspension was then mixed thoroughly and loaded into a McMaster counting chamber and the number of eggs in the grid area was counted. The EPG was noted as the number of eggs in 1 gram of faeces.

Generalized *H. contortus* specific IgG immune responses were evaluated by using an indirect enzyme-linked immunosorbent assay (ELISA). To obtain crude somatic antigens, 200 000 *H. contortus* L3 larvae were thoroughly crushed and kept at 4 ℃ overnight in the PBS. The mixture was then centrifuged at 12 000 × g for 20 minutes at 4 ℃. The supernatant was collected, and the protein concentration was measured using Pierce BCA protein Assay kit (ThermoFisher Scientific, Waltham, USA) and stored at -20 ℃ until further use.

The ELISA was undertaken using the technique described by Schallig, Hornok and Cornelissen (1995). Briefly, flat bottomed 96 well plates were coated with 50 µL of 10 µg/mL crude *H. contortus* larval antigen in coating buffer. The plates were incubated overnight at 4 ℃. The plates were washed three times with wash buffer (0.05 percent tween 20 in PBS). Then, the wells were filled with 200 µL of 5 percent w/v skimmed milk powder in PBS. The plates were then incubated for 1 h at room temperature followed by three washes. The wells were then filled with 50 µL of 1:10 diluted (diluted in blocking buffer) serum and incubated at room temperature for 2 h. The plates were washed after 2 h and filled with 50 µL of rabbit anti goat HRP antibody diluted 1:2000 in the blocking buffer. The plates were incubated at room temperature for 1 h. Again, the plates were washed three times with wash buffer and finally 50 µL TMB substrate buffer were added and the plates were incubated at 37 ℃ for 10 minutes. Then 50 µL of H_2SO_4 was added to stop the reaction and the optical density value was read at 450 nm using the microplate reader.

Evaluation of protective immune responses following vaccination

A second group of 20 kids (4 months of age) were recruited for the study of evaluating protective immune responses for the irradiated (200 Gy) *H. contortus* L3 larval vaccine. The kids were assigned to four groups of five kids each, with each group defined according to whether the kids were vaccinated and challenged (Table 1). Faecal samples were collected weekly for monitoring the EPG levels.

All animals were subject to postmortem analysis. The abomasal mucosa was scraped and the contents were digested using pepsin in the presence of 1 percent HCL and the mixture was incubated at 37 ℃ for 3 hours. The final volume of the suspension was set as 500 mL. The

Table 1: Vaccination and challenge study design

Group	Day 0	Day 35	Day 63	Day 180
1	Vaccine 1*	Vaccine 2*	Challenge**	Post-mortem
2	Vaccine 1*	Vaccine 2*	None	Post-mortem
3	None	None	Challenge**	Post-mortem
4	None	None	None	Post-mortem

Source: Authors' own elaboration.

Note: * *H. contortus* irradiated L3 larval vaccine (200 Gy irradiation dose) 10 000 administered orally; ** *H. contortus* infective L3 larvae 10 000 administered orally.

mixture was strained through a tea strainer. Then the filtrate was centrifuged at 800 × g for 10 minutes. For the sediments, 25mL of normal saline was added with 2 drops of iodine. Total number of larvae was counted in ten 1 mL aliquots under dissecting microscope.

3. Results

Vaccine development and irradiation dose testing

Figure 1 shows the daily trends in EPG of the four kids administered *H. contortus* L3 larvae for the preparation of the vaccines. Some variability among animals was observed, but in general eggs began to appear in the faeces 14 days of infection and the infections persisted until 84 days.

Figure 2 shows the trends in mean PCV among control animals and those treated with vaccines developed with different irradiation doses. No change of PCV was observed for the control group, while there was PCV reduction from Days 14 to 63 in the vaccinated groups, indicating infection. However, the irradiation dose of 200 Gy demonstrated the lowest PCV reduction.

Similarly, the total RBC counts decreased from Day 35 to Day 77 following vaccination in groups receiving the 50 Gy and 100 Gy vaccines, while the 200 Gy group had very low reduction compared to uninfected controls (Figure 3). This finding indicates the lower rate of infection in the 200 Gy group following the vaccination. Thus, when irradiated with a 200 Gy irradiation dose the rate of infectivity of L3 larvae is minimized, reducing the pathological changes.

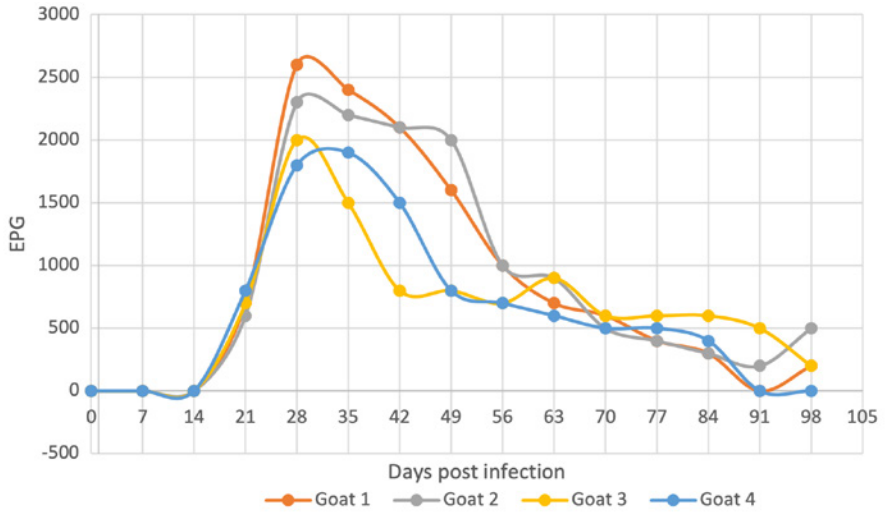
In all groups where the vaccine was given, there was increase in mean absolute eosinophil count after 77 days of vaccination, indicating the induction of eosinophil mediated immune responses in comparison with the control group (Figure 4).

Similar results were demonstrated in mean absolute lymphocyte counts, in all vaccinated groups when compared with the unvaccinated group (Figure 5). This is a good indication that the L3 larval vaccine showed increased lymphocyte mediated immune responses at all used irradiation doses.

If the irradiated L3 larval vaccine provides protection against the *H. contortus* infection, the EPG should have been minimal in vaccinated animals. Accordingly, in the 200 Gy group EPG was very low. However, at irradiation doses of 50 Gy and 100 Gy, EPS was increased, simulating natural infection among the goats (Figure 6). Thus, it could be concluded that the vaccine made with 200 Gy irradiation of *H. contortus* L3 larvae had the highest success rate.

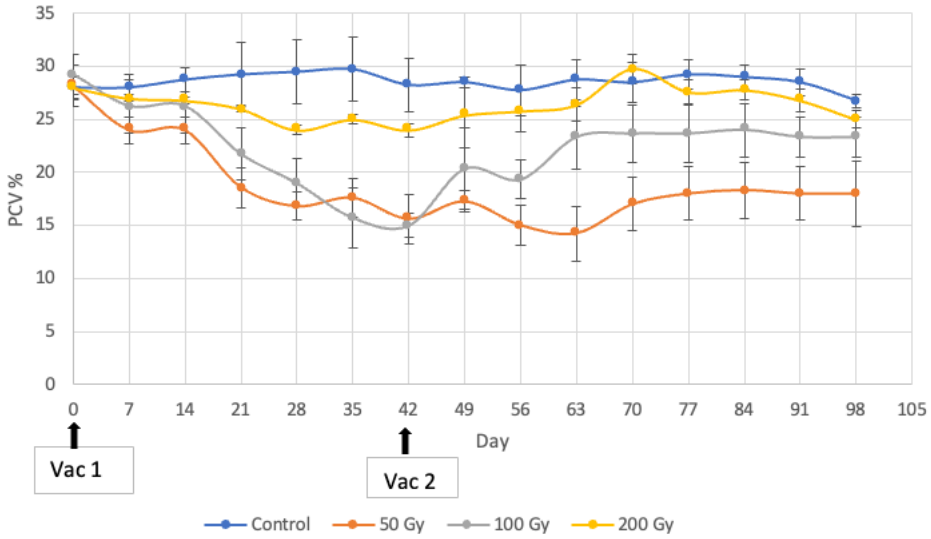
The total protein concentration in plasma is one of the important measurements to evaluate the loss of proteins due to enteropathy caused by the blood sucking nature of the adult *H. contortus* worms. In comparison with the control group, minimal reduction of the total protein concentration was observed in the 200 Gy group. However, the 50 and 100 Gy groups demonstrated greater reductions in total protein concentration, mimicking natural *H. contortus* infection (Figure 7).

As previously mentioned, the generalized humoral immune responses against *H. contortus* irradiated larval vaccine was evaluated by IgG ELISA. The three groups that received the vaccine developed increased *H. contortus* specific IgG responses, which could be detected by examining the weekly collected serum samples. In comparison to the control group there was a significantly increased IgG response detected for all three vaccines tested. However, the level of IgG against *H. contortus* larval antigen did not show a significant increase following the second vaccine at day 42; the levels were maintained (Figure 8). The *H. contortus* specific IgG concentrations were maximum when the irradiation dose of 200 Gy was used, indicating that the vaccine made using 200 Gy irradiation dose was the best vaccine.



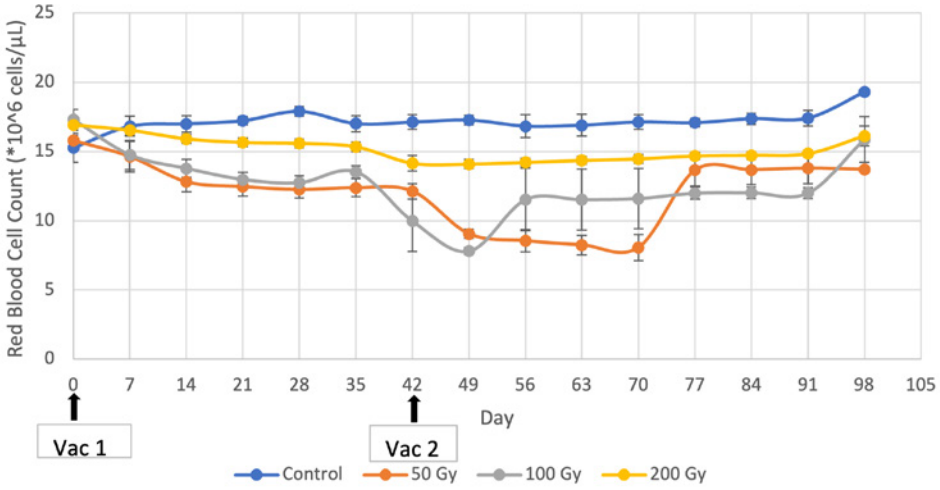
Source: Author's own elaboration.

Figure 1: Eggs per gram of faeces (EPG) measured at 7-day intervals in four goats following infection with *H. contortus* 10 000 L3 larvae



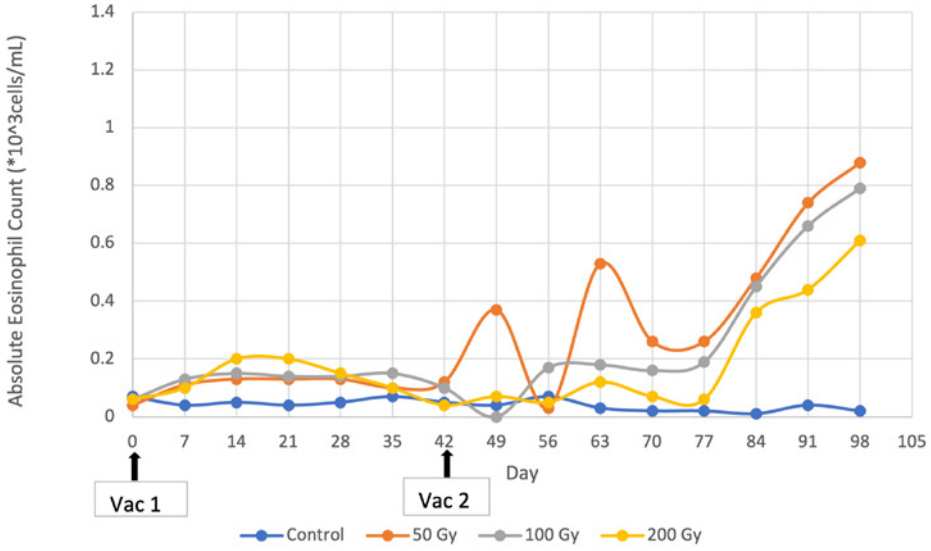
Source: Author's own elaboration.

Figure 2: Mean PCV measured at 7-day intervals in four treatment groups following vaccination



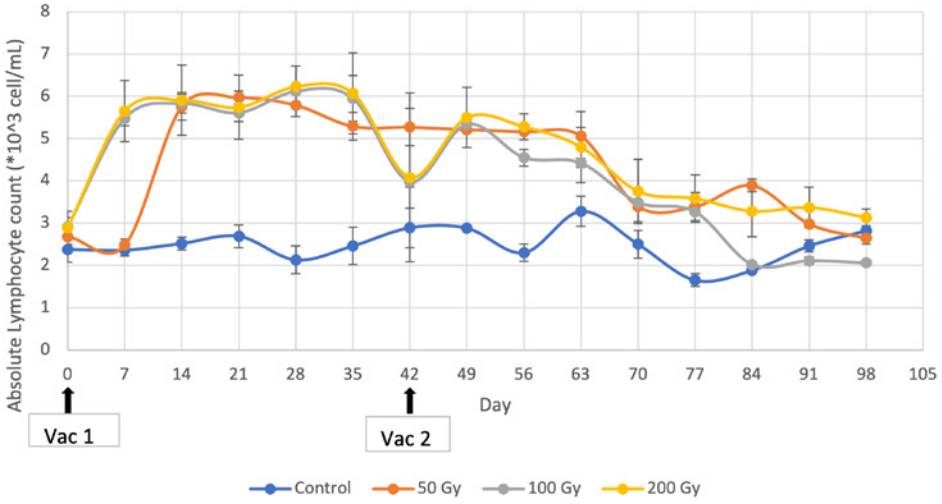
Source: Author's own elaboration.

Figure 3: Mean red blood cell counts measured at 7-day intervals in four groups following vaccination



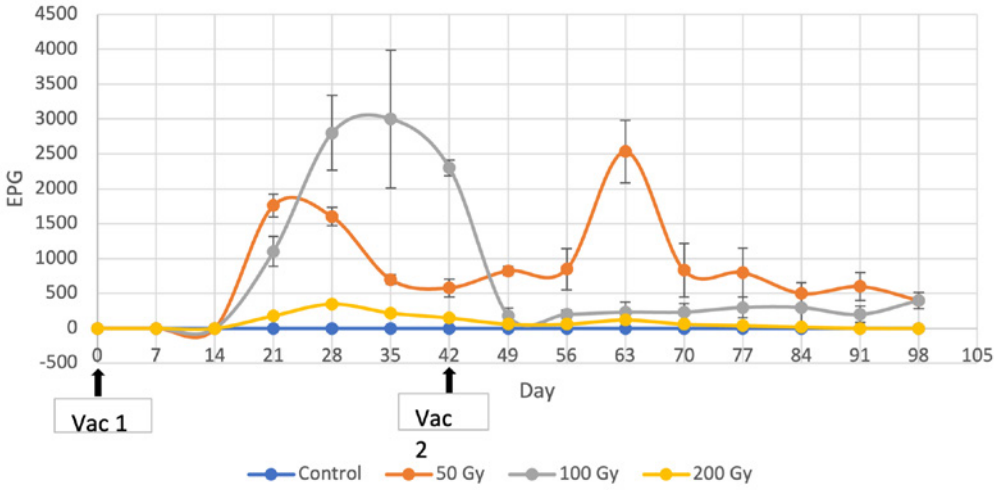
Source: Author's own elaboration.

Figure 4: Mean absolute eosinophil counts measured at 7-day intervals in four treatment groups following vaccination



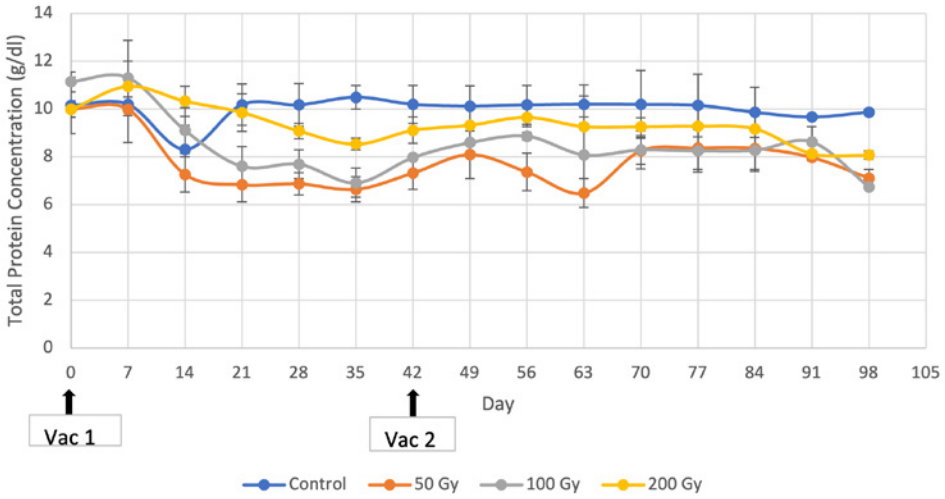
Source: Author's own elaboration.

Figure 5: Mean absolute lymphocyte counts measured at 7-day intervals in four groups following vaccination



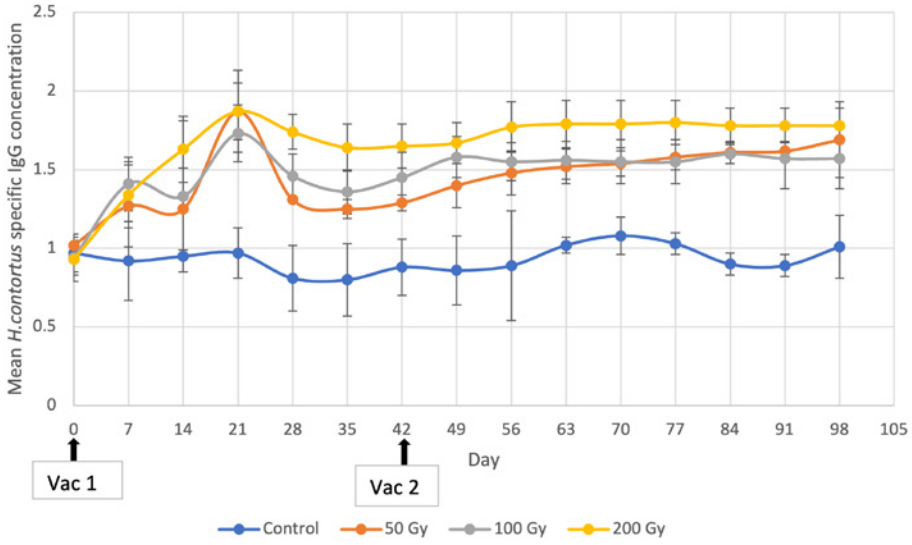
Source: Author's own elaboration.

Figure 6: Weekly egg per gram of faeces (EPG) of vaccinated and non-vaccinated groups



Source: Author's own elaboration.

Figure 7: Mean total protein concentration of vaccinated and non-vaccinated groups



Source: Author's own elaboration.

Figure 8: *H. contortus* crude antigen specific IgG concentrations of serum in vaccinated and non-vaccinated groups measured at 7-day intervals

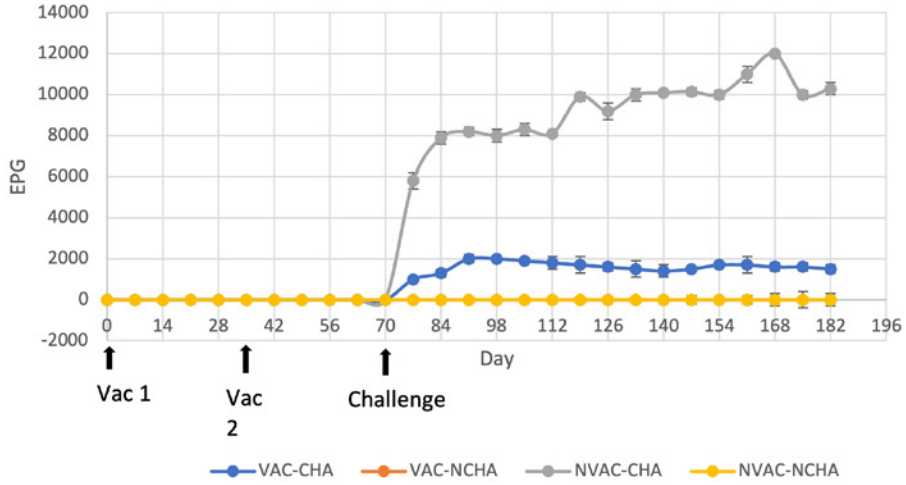
Evaluation of immune response following vaccination

In the study to test the immune response against the 200 Gy vaccine (Figure 9), the EPG was elevated exclusively in the challenge only group, while EPG was zero in groups where the challenge was not given. Low (but detectable) EPG was maintained among the kids that were administered two vaccine doses and then challenged with *H. contortus* infective larvae, indicating the development of protective immune responses (Figure 9).

In all four groups, total worm burden in the abomasum was evaluated and the adult worm counts demonstrated the highest prevalence of adult worms in the challenge only kids (Table 2). Lower counts were observed among the group where two doses of vaccines were administered. This result demonstrates that the L3 larval vaccine prepared with a 200 Gy irradiation dose induced sufficient protective immune responses over a period of 117 days. Furthermore, the tissue larval counts demonstrated a very high proportion of arrested larvae in the vaccinated and challenged group compared to challenge only group (Table 2).

4. Discussion

An irradiated larval vaccine against *H. contortus* was developed using L3 larvae. The generalized immune responses were evaluated in terms of *H. contortus* L3 larval crude antigen specific IgG, EPG and haematological parameters. The study objectives included the determination of the best irradiation dose for the L3 infective larvae to obtain maximum immunogenicity. Three irradiation doses used were 50 Gy, 100 Gy and 200 Gy. Further, the study aimed to assess the development of protective immunity following vaccination and challenge study.



Source: Author's own elaboration.

Figure 9: Mean eggs per gram of faeces (EPG) of goat kid groups vaccinated or not vaccinated with 200 Gy irradiated *H. contortus* larval vaccine (VAC or NVAC) and challenged or not challenged with L3 infective larvae (CHA or NCHA)

Table 2: Adult worm counts at day 182 from all vaccinated and challenged groups of goat kids

Group	Day 0	Day 35	Day 63	Adult worm counts	Tissue larvae counts
1	Vaccine 1*	Vaccine 2*	Challenge**	2 489 ± 38	14 356 ± 1 291
2	Vaccine 1*	Vaccine 2*	None	218 ± 68	12 458 ± 728
3	None	None	Challenge**	5 972 ± 1 939	103 ± 34
4	None	None	None	0	0

Source: Authors' own elaboration.
Note: * *H. contortus* irradiated L3 larval vaccine (200 Gy irradiation dose) 10 000 administered orally; ** *H. contortus* infective L3 larvae 10 000 administered orally.

The parasitic effect on the host is mainly denoted by the EPG, which is a measure of number of adult worms in the host and the worm fecundity. The difference of EPG is thus of very high importance for demonstrating immunity (Nayebzadeh, Seyfiabadshapouri and Hoghooghi, 2005). The current study showed lower EPG in the 200 Gy group than in the 50 Gy and 100 Gy groups, demonstrating the smallest infection rate and highest IgG activity. This finding in vaccinated goats is in agreement with Aboelhadid *et al.* (2013), where UV irradiation was used to irradiate the L3 larvae. In their study, exposure of L3 larvae to UV light for 60 minutes showed lower faecal egg counts compared to UV exposure of 30 minutes. They also observed that the eggs in the faeces were deformed, which was not observed in the current study (Aboelhadid *et al.*, 2013). The EPG of vaccinated and challenged goat kids in the current study demonstrated the development of protective immune responses following vaccination. Interestingly, the EPG of vaccinated and challenged animals was reduced 3–4 fold relative to the challenged only animals. This observation continued throughout the entire experimental period of 117 days following infective L3 larval challenge.

Monitoring serum IgG concentration demonstrated the generalized immune response induction against the irradiated *H. contortus* L3 larval vaccine. At days 28 and 35 we observed reduced IgG concentrations, indicating rapid reduction of immune response. Thus the second vaccine was administered to keep constant IgG concentrations for a longer period of time. In the production of irradiated vaccines against parasitic diseases, many studies have demonstrated that two doses of vaccines are needed for immune response development (Jian *et al.*, 2006; Ali *et al.*, 2007). For example, two doses of irradiated *Dictyocaulus filaria* vaccine were given 4 weeks apart to develop effective immunity in lambs (Jarrett *et al.*, 1958).

A study by Aboelhadid *et al.* (2013) showed that the UV radiation of L3 larvae and subsequent vaccination prevented larval development from the L3 to L4. The arrest in this development was shown to be dose dependent. Therefore, we can assume that the lower EPG detected in the 200 Gy group may be due to developmental arrest of the L3 larvae in the abomasum (Aboelhadid *et al.*, 2013). The current study demonstrated greatly reduced larval development in the vaccinated goat kids. These larvae may play a major role for the long-lasting development of protective immune responses.

The haematological parameters examined in the vaccinated and unvaccinated goats showed significant reduction in PCV, total RBC, total serum protein in 50 and 100 Gy vaccinated groups. In cases of natural infection by *H. contortus*, significant changes in PCV, RBC count and total serum protein could be observed (Alam, Hassanen and El-Mandrawy, 2020). The data of this study indicates when irradiation doses of 50 Gy and 100 Gy were used, the attenuation acquired is not sufficient and thus the haematological parameters aligned with natural infection. However, for all three irradiation doses, *H. contortus* specific IgG, absolute eosinophil counts and absolute lymphocyte counts were very high compared to the control group. Thus, the vaccination with irradiated L3 larvae induced immunity to a certain degree in all goats. However, to further clarify this observation, a group of naturally infected goats in the field should have been monitored.

5. Conclusions

The findings of this study lead us to conclude that the 200 Gy irradiation dose is ideal for the production of *H. contortus* L3 larval vaccine for goats. Two doses of vaccines at day 0 and day 42 provided high humoral immunity measured by *H. contortus* L3 crude antigen specific IgG and eosinophil and lymphocyte mediated cellular responses. When vaccinated and challenged with L3 infective larvae, a three-to-four-fold reduction in EPG was observed, ensuring the protective immune responses induced by the irradiated *H. contortus* larval vaccine. Thus, irradiated *H. contortus* L3 larval vaccine has high potential for controlling *H. contortus* infection among goats.

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Transboundary animal and zoonotic diseases

WOAH's Programmes to prevent emerging zoonotic diseases

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Abstract

The World Organisation for Animal Health (WOAH) is the inter-governmental organization charged with improving animal health and welfare worldwide. WOAH's standards are recognised by the World Trade Organization (WTO) as reference international sanitary rules. In WOAH's programme of work there are global initiatives to address endemic and emerging zoonotic diseases, and core programmes to reduce and manage the risk from the emergence of new ones. In this paper, we provide an overview of important zoonotic diseases, WOAH's programmes and its work with partners to prevent them and mitigate their consequences in a post-COVID world.

1. The WOAH

The need to halt the global spread of rinderpest led to the creation of the Office International des Epizooties nearly 100 years ago (WOAH, 1924). In 2003, the Office International des Epizooties became the World Organisation for Animal Health but kept its historical acronym OIE. The WOAH presently has 182 Members and 13 regional and subregional representations, in addition to its headquarters in Paris. WOAH's standards cover trade of animals and animal products, control of diseases, animal welfare, diagnostic tests and vaccines, among other topics. These standards are recognized by the WTO as the reference for international sanitary rules (WTO, 1994). The WOAH works to provide its members with transparent, timely and accurate information on animal disease events, and prides itself on the scientific excellence of its network of more than 240 Reference Centres.

2. Zoonotic diseases

Zoonotic diseases, or zoonoses, are infectious diseases of vertebrate animals that can be transmitted to humans, and vice-versa, either through direct contact or indirectly, through insect or arthropod vectors, contact with contaminated environments, or consumption of contaminated food and water (Wang and Crameri, 2014). Addressing zoonotic disease emergence is of utmost importance to safeguard human health. More than half of the existing human diseases are zoonotic, and the majority of the newly emerging diseases, such as novel strains of influenza, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), novel viral haemorrhagic fevers, and others, are of animal origin (Jones *et al.*, 2008).

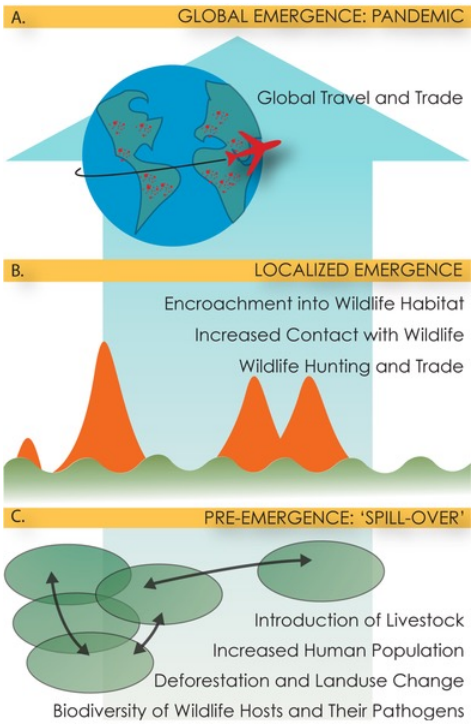
Emergence mechanisms

Human demographic expansion, growth of intensive farming systems, enlargement of crop lands, and the increased development of human settlements and cities have contributed to the disruption of natural ecosystems through human encroachment into what once were wild areas (Jones *et al.*, 2008). This results in increased opportunities for previously unknown pathogens to emerge, by "spilling-over" from their animal hosts and spreading within the human population.

Historically, the emergence of new human diseases from animal sources was associated with major societal change. However, in the current paradigm where global trade and international travel are the norm, we see more frequent contacts among humans and domesticated and wild animals. Animal populations that were once confined to their own ecological niche are now in direct contact with humans and with animals foreign to their natural habitat,

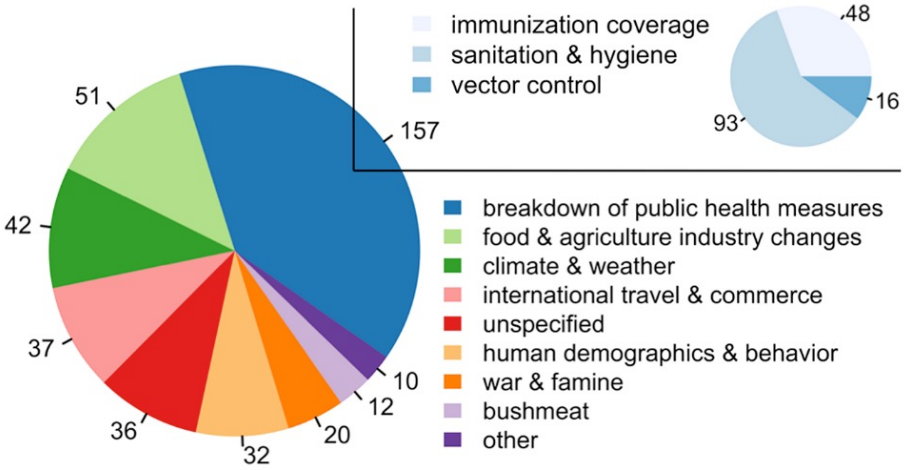
creating opportunities for disease spill-over (Bogich *et al.*, 2012). As shown in part B of Figure 1 (Bogich *et al.*, 2012), numerous new diseases that emerge locally (green waves) end-up fading-off. However, the present-day hyper-connected environment acts as an accelerator, increasing the number of opportunities for pathogens to be carried long-distance around the globe in an ever-shortening time span. If a pathogen is successful in infecting and being transmitted among humans, there is the potential for a local emergence to spread globally (red waves) and take on pandemic proportions, as seen in 2020 with SARS-CoV-2.

When staving-off the progress of the local emergence of new diseases, strong health systems are key. A recent publication from Bogich *et al.* (2012) illustrated by Figure 2 shows that, out of the 397 outbreaks whose attribution was analysed, 157 were associated with the breakdown or absence of public health infrastructure, among which, most importantly, of sanitation and hygiene measures. Global health systems are only as strong as their weakest link. Next in the list of attributions were food and agriculture industry changes, whose impact in the spill-over of zoonotic agents and local emergence of new diseases was described above.



Source: Bogich, T.L., Chunara, R., Scales, D., Chan, E., Pinheiro, L.C., Chmura, A.A., Carroll, D., Daszak, P. & Brownstein, J.S. 2012. Preventing pandemics via international development: A systems approach. *PLoS Medicine*, 9(12): e1001354. https://doi.org/10.1371/journal.pmed.1001354

Figure 1: Preventing pandemics via international development: a systems approach



Source: Bogich, T.L., Chunara, R., Scales, D., Chan, E., Pinheiro, L.C., Chmura, A.A., Carroll, D., Daszak, P. & Brownstein, J.S. 2012. Preventing pandemics via international development: A systems approach. *PLoS Medicine*, 9(12): e1001354. https://doi.org/10.1371/journal.pmed.1001354

Figure 2: Preventing pandemics via international development: a systems approach

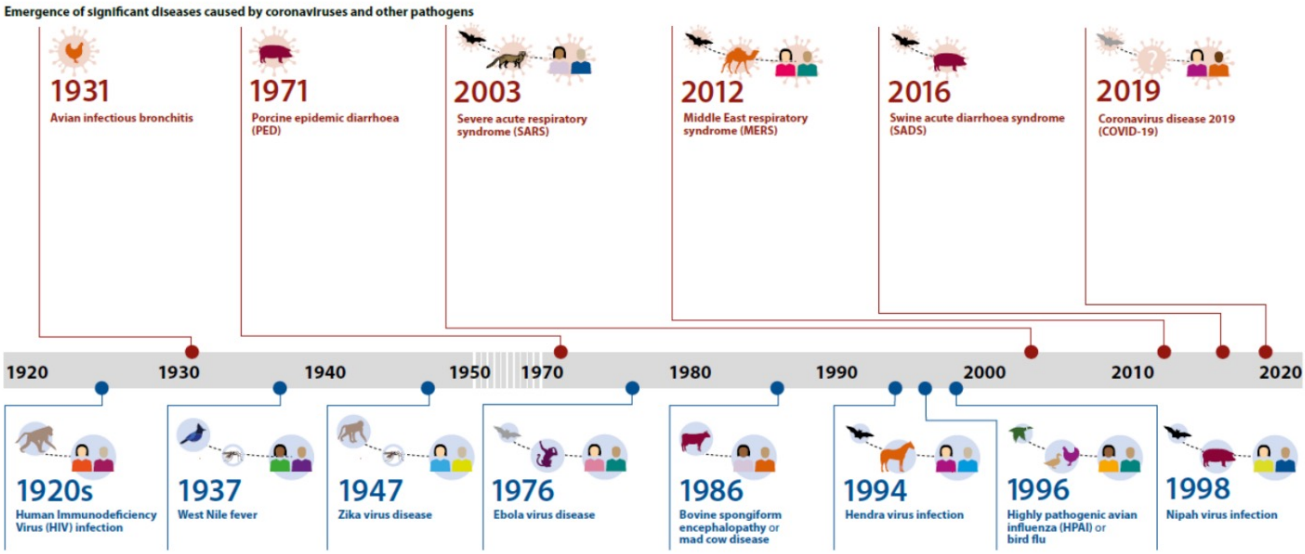


Figure 3: Timeline depicting the emergence of new zoonotic diseases caused by coronaviruses (on top) and other agents (on the bottom); adapted from: United Nations Environment Programme and International Livestock Research Institute (2020); Preventing the Next Pandemic: Zoonotic diseases and how to break the chain of transmission; Nairobi, Kenya

Recently emerged zoonoses

The better understanding of the risk factors that drive and accelerate emerging pathogens, combined with an improvement in the laboratory techniques for identification of microorganisms, resulted in the detection of growing number of new emerging zoonoses in the last 100 years.

Figure 3 is a timeline depicting the emergence of new zoonotic pathogens since the beginning of the 20th century (UN Environment Programme and International Livestock Research Institute, 2020). The top row shows zoonotic diseases caused by coronaviruses, while the bottom row shows an array of viruses and one prion. Next to each new pathogen there are a couple of small bubbles describing the animal host species and, in some cases, the intermediary animal host before spill-over from animals to human occurred. It is worth noting that the majority of new emerging zoonoses are caused by viruses, while many of the endemic zoonoses covered in the next section are caused by bacteria.

Endemic zoonoses

The WOAH works with its partners to implement Global Initiatives for control and eradication of certain animal diseases within its member countries. Many of these diseases are endemic zoonoses that are well established in the human population and have varied causative agents. Some of the zoonotic diseases for which the WOAH has a strategy and active partnerships either at tripartite level (with the Food and Agriculture Organization of the United Nations, FAO, and the World Health Organization, WHO) or with other international organizations and research institutes are influenza, bovine tuberculosis, rabies and viral haemorrhagic fevers. For surveillance in general, the WOAH collaborates with FAO and WHO through GLEWS+, the Global Early Warning System for health threats and emerging risks at the human-animal-ecosystems interface.

Zoonotic Influenza

The global spread of H5N1 avian influenza in the early 2000s intensified the joint work of the Tripartite on this matter. Since then, the three organizations regularly exchange information on the global situation of animal influenza as one of their priority topics under Tripartite plan of work.

One of the main initiatives related to zoonotic influenza is the joint WOAH-FAO network of expertise on animal influenza, OFFLU. FAO and WOAH jointly manage OFFLU, which works to reduce the negative impacts of animal influenza viruses. OFFLU promotes collaboration between animal health experts and the human health sector (Pavade, 2020). OFFLU was launched in 2005 to support the control of avian influenza, and it was expanded in 2009 to include all animal influenzas and to further support Veterinary Services in their efforts to reduce risks to animals and the public from animal influenza viruses. Members of the OFFLU network exchange scientific data and biological materials and offers technical advice and veterinary expertise to Members. Very importantly, OFFLU and the WHO networks collaborate closely to contribute to risk assessment and the early preparation of human vaccines against influenzas that may emerge from animals, thus posing a pandemic threat.

In addition to collaborating with partners, the WOAH draws on its network of expertise to produce analyses of the current global situation for avian influenza, the frequency of which is driven by the number and severity of notifications for avian influenza received through WOAH-WAHIS from WOAH Members.

Rabies

Rabies is one of the best known and deadliest zoonoses. Rabies is still present in two-thirds of the worlds' countries and it kills nearly 59 thousand people per year worldwide, most of whom are children in low-income countries (Hampson *et al.*, 2015). Around 99 percent of human cases of rabies are due to dog bites. Unlike many other zoonotic diseases, rabies is preventable and tools for its eradication are already available. The WOAH does significant work on advocacy, communications, capacity building and training to promote dog vaccination as a way to eradicate dog-mediated human rabies. The WOAH has a rabies vaccine bank available to its members, which ensures that good quality and affordable rabies vaccines are delivered in a timely manner to those who need them. The WOAH also has in place laboratory twinning projects dedicated to rabies to improve the capacity of its Members to perform diagnostics and surveillance for rabies in the regions that need them the most. Finally, the WOAH has partnered-up with WHO, FAO and the Global alliance for rabies control (GARC) to set the goal of eradicating dog-mediated human rabies by 2030.

Viral Haemorrhagic Fevers

Since 2017 the WOAH has been leading the implementation of the EBO-SURSY project (WOAH, 2021). This project, which is funded by the European Union, aims to strengthen the national and regional early detection systems for wildlife in West and Central Africa using a "One Health" and multi-sectoral approach. The objective is to foster the capacity to better detect, differentiate, and prevent future outbreaks of Ebola virus disease and four other viral haemorrhagic fevers: Marburg virus, Rift Valley fever, Crimean-Congo haemorrhagic fever and Lassa fever. The project's activities include trainings, communication, twinning, participatory surveillance, data collection and analyses. The WOAH's work on the EBO-SURSY project is made possible by a close, collaborative relationship with its project partners: CIRAD, IRD and Institut Pasteur.

Zoonotic Tuberculosis

Zoonotic tuberculosis is a form of the disease in humans caused by *Mycobacterium bovis*, which belongs to the Mycobacterium tuberculosis complex. Within animal populations, *M. bovis* is the causative agent of bovine tuberculosis (WHO, FAO and WOAH, 2017). It mainly affects cattle, which are the most important animal reservoir, and can become established in wildlife. The disease results in important economic losses and trade barriers with a major impact on the livelihoods of poor and marginalized communities. Globally, in 2016 there were around 150 000 cases detected (WHO, FAO and WOAH, 2017). The number may appear low from a global perspective; however, Zoonotic tuberculosis presents many detection challenges, and its burden is likely underestimated. In many instances, *M. bovis* is not possible to differentiate from *M. tuberculosis* when using simple diagnostic tests. Therefore, there is a high likelihood of misdiagnosis. Moreover, since zoonotic tuberculosis is usually extrapulmonary, the same drugs used to treat *M. tuberculosis* do not work in *M. bovis* (WHO, FAO and WOAH, 2017).

The WOAH, WHO, FAO and the International Union Against Tuberculosis and Lung Disease jointly launched the first-ever roadmap to tackle zoonotic TB in October 2017, which is based on a One Health approach.

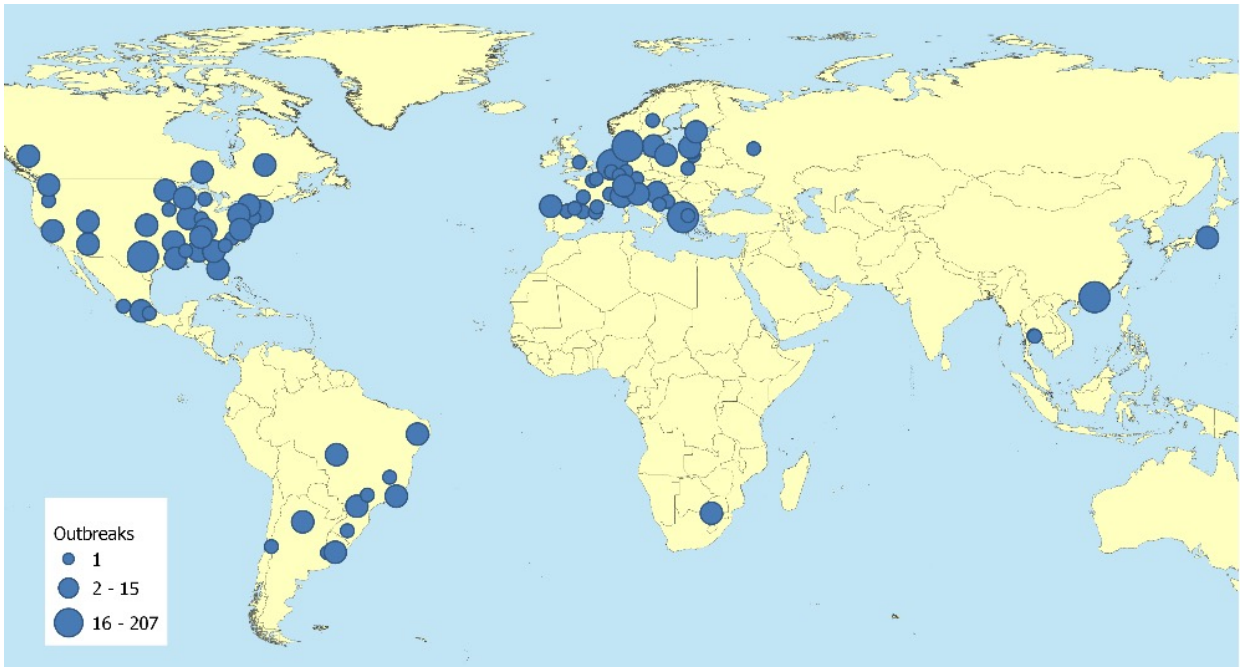
3. Post-COVID world

The COVID-19 pandemic was a watershed moment not only for the global health community, but for the human population in general. COVID-19 has changed the way the WOAH works and shifted some of the organization's priorities. The WOAH response to COVID-19 started early, in January 2020. WOAH immediately engaged with its Tripartite partners and convened an *ad hoc* group of experts to advise and to assist in compiling guidance to WOAH Members with the latest scientific information, e.g. the susceptibility of animal species and their role in transmission. Since then, two other groups of experts – the *ad hoc* Group on Safe Trade in Animals and Animal Products and the Advisory group on SARS-CoV-2 Evolution in Animals – have been convened and all three groups meet as needed. With the help of these groups, the WOAH has developed guidance documents for its Members related to COVID-19 at the human-animal interface.

WOAH Members are asked to report cases of SARS-CoV-2 infection in animals through our WOAH-WAHIS. The map in Figure 4 shows the total number of animal outbreaks reported globally since the beginning of the COVID-19 pandemic until 31 July 2021. The distribution of these outbreaks is not only related to the types of animal populations present in each country (e.g. mink farms and zoos) but also to the existence of laboratory capacity to detect these outbreaks.

Future work

COVID-19 has also influenced future areas of work at the WOAH. In response to an evolving landscape, and the needs expressed by its Members, the WOAH has developed a framework aimed at improving wildlife health by embracing a One Health approach – the WOAH Wildlife Health Framework. It was developed in a collaborative manner in 2020, and includes inputs from Members, collected through a survey, and from the Working Group on Wildlife, WOAH staff and international conservation organizations. It is also informed by lessons learned during the EBO–SURSY Project, which was mentioned during the section on viral haemorrhagic



Notes: Final boundary between the Sudan and South Sudan has not yet been determined. Dotted line represents approximately the Line of Control in Jammu and Kashmir agreed upon by India and Pakistan. The final status of Jammu and Kashmir has not yet been agreed upon by the parties.

Source: WOAH. 2020. COVID-19 -- Map: cases of SARS-CoV-2 infection in animals reported to WOAH since March 2020. In: WOAH.org. [Cited 29 March 2023.] https://www.woah.org/en/what-we-offer/emergency-preparedness/covid-19/#ui-id-3

Figure 4: Reports of infection of animals with SARS-CoV-2 received from WOAH Members as of 31 July 2021

fevers. The Wildlife Health Framework aims to protect wildlife health globally to achieve One Health. Two main priorities have been identified and will contribute to public health and conservation objectives: 1) to improve WOAH Members' ability to manage the risk of pathogen emergence in wildlife and transmission at the human–animal–ecosystem interface, while taking the protection of wildlife into account; and 2) to support WOAH Members to improve surveillance systems, early detection, notification and management of wildlife diseases.

4. Conclusions

COVID-19 has brought One Health to the attention of the highest decision fora. Zoonosis and the human-animal-ecosystems interface are an expanding area of work, and it is critical that the interest in these topics does not fizzle out as the world recovers from the present pandemic.

At the 2021 General Session of the World Assembly of WOAH Delegates, a Resolution called "How the WOAH can support Veterinary Services to achieve One Health resilience" was endorsed. The Resolution stems from a technical item with a matching name and emphasises WOAH Members' support to the initiatives the WOAH has underway for disease prevention and control, and support of national veterinary services such as the wildlife health framework, multilateral partnerships, emergency management, sustainable laboratories initiatives, and data management among others. All this is in line with WOAH's mission to safeguard animal health and welfare and, more specifically, its activities to prevent emerging zoonotic diseases.

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Immune responses to Foot-and-Mouth Disease vaccines in a guinea-pig model

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Abstract

Vaccination of cattle against Foot-and-Mouth Disease (FMD) is a well-established strategy for control of the disease. In this research, gamma irradiated FMD virus type O/IRN/2010 was used as an inactivated irradiated vaccine (GIV), and a DNA vaccine was constructed based on the VP1 gene and co-administrated with a molecular adjuvant. These vaccines were compared to a conventional inactivated vaccine. The study was carried out on seven twice-vaccinated guinea pig groups and one negative control group. The immune responses of vaccinated groups were significantly greater than the negative control group ($P < 0.05$). The neutralizing antibody titration showed the highest humoral immunity in homologous GIV (Gamma Irradiated Vaccine) and conventional vaccine groups ($P < 0.05$), and confirmed protection for three heterologous prime-boost (PB1, PB2 and PB3) groups. Also, the proliferation of spleen T lymphocyte was increased significantly in PB1, PB2, PB3 and DNA vaccine+GMCSF groups, as indicated by the Stimulation Index (SI). PB1 and PB2 groups showed a significant increase for Th1 cytokine concentrations. The PD50 of six challenged vaccine groups (i.e. DNA vaccine+GMCSF, GIV, Conventional vaccine, PB1, PB2 and PB3) were 4.46, 7.08, 6.20, 7.08, 6.91 and 6.28, respectively. A heterologous prime boost strategy with DNA vaccine+GMCSF and inactivated vaccine was found to be the best method for inducing humoral and cellular immunity and increasing Th1 cytokines.

Keywords

Foot-and-Mouth Disease virus, vaccine, gamma irradiated, immune responses

1. Introduction

Prevention and control of disease is the main aim of livestock vaccines. Foot-and-Mouth Disease (FMD) is a severe contagious disease in cloven-hoofed animals (Kahn *et al.*, 2002). There are seven serotypes for this virus, and three of them are in the Islamic Republic of Iran. Nowadays, an inactivated vaccine is used for the prevention of this disease. Some disadvantages of the inactivated vaccine are short shelf life, chemical residues in the final product, viral escape and a long inactivation time (Viljoen and Luckins, 2012). The FMD virus (FMDV) RNA genome has a single large open reading frame encoding a polyprotein that can generate four structural proteins VP1, VP2, VP3, and VP4 and ten non-structural proteins. The VP1 protein is exposed on the capsid surface and carries the virus major neutralizing antigenic sites. The VP1 coding sequence has been used in the development of engineering vaccines and the establishment of diagnostic methods for typing and subtyping of the virus (Zhang *et al.*, 2015). The main aim of this research was to compare the efficacy of an inactivated irradiated FMD vaccine prepared by using gamma irradiation, a DNA vaccine based on VP1 gene and conventional vaccine. We evaluated the humoral and cellular immune responses to these three vaccines with a guinea pig model and compared the responses with both homologous and heterogenous "Prime-boost" vaccination.

2. Materials and methods

Virus and vaccines

To create the irradiated vaccine, FMD virus type O/IRN/2010 was multiplied on a BHK_{21} cell line and irradiated with different doses of gamma rays. Dose/responses curves were drawn by Origin software, and the D_{10} Value and the optimum dose of gamma irradiation for complete inactivation were calculated. Antigenic characteristics of irradiated and un-irradiated viral samples were compared using the complement fixation method, and a safety test for the inactivated virus was confirmed by four blind passages cell culture on an IBRS2 cell line. The inactivated virus was then formulated as a gamma irradiated vaccine (GIV) plus Al $(OH)_3$ and saponin as adjuvants (Sólyom *et al.*, 1980; Smolko and Lombardo, 2005; Motamedi-Sedeh *et al.*, 2015; Zhang *et al.*, 2015; Rathogwa *et al.*, 2021).

To prepare the DNA vaccine, the VP1 gene was also sub-cloned into the unique *Kpn* I and *Bam*H I cloning sites of the pcDNA3.1+ vector. The VP1 nucleotide sequence data of FMDV type O/IRN/2010 had been previously deposited in the GenBank database under the accession number JN676146. A sub-cloned PCMV-SPORT vector with GMCSF (Granulocyte Monocyte Colony Stimulating Factor) gene that was gifted from the Pasture Institute of Iran was administered as a molecular adjuvant with the DNA vaccine (Knudsen, Groocock and Andersen, 1979; Kim *et al.*, 2006).

A conventional vaccine was prepared by binary ethylene imine (BEI) inactivation (Smolko and Lombardo, 2005; Motamedi-Sedeh *et al.*, 2015).

Vaccination schedule

Guinea pigs were chosen as the experimental model in this research because of the similarities of clinical symptoms to those of swine and cattle. Unlike swine or cattle, guinea pigs cannot be infected via respiratory aerosols. However, inoculation with virus into the skin or tongue leads to clinical infection (Smolko and Lombardo, 2005).

Sixty-three female guinea pigs (250–300 g weight) were assigned to nine groups: 1) the sub-cloned pcDNA3.1+VP1 gene cassette as the DNA vaccine (100 µg); 2) DNA vaccine (50 µg) plus PCMV-SPORT-GMCSF vector (50 µg) as a molecular adjuvant; 3) GIV (200 µl); 4) conventional vaccine (200 µl); 5) Prime boost1 (PB1) – DNA vaccine (50 µg) plus the PCMV-SPORT-GMCSF vector (50 µg) as the primary vaccine and the conventional vaccine (100 µl) as the booster vaccine; 6) PB2 – DNA vaccine (50 µg) plus PCMV-SPORT-GMCSF vector (50 µg) as the primary vaccine and GIV(100 µl) as the booster vaccine; 7) PB3 – GIV (200 µl) as the primary and DNA vaccine (25 µg) plus PCMV-SPORT-GMCSF vector (25 µg) as the booster vaccine; 8) Phosphate buffered saline (PBS) (100 µl) as a negative control; and 9) a pre-immune group from which the blood and spleen samples were evaluated before vaccination.

Vaccination was done in two doses, separated by a 21-day interval infra-auxiliary. Two weeks post-vaccination we collected blood and spleens from all vaccinated animals for testing of immune response.

For this research, we followed all institutional and national guidelines on animal care, which had been adopted from the Horizontal legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU as amended by Regulation (EU) 2019/1010). The procedures used were approved for implementation by the Tehran University of Medical Science.

Post-vaccination monitoring to assess immunity

Neutralizing antibody titration was determined by the serum neutralization test (SNT), and proliferation of splenic T lymphocytes was measured by the MTT test (Cell proliferation kit 1, Roche, Cat No: 11-465-007-001). Cytokine assays were done by using ELISA kits for interferon gamma (IFN-γ) and several interleukins (IL-2, IL-4 and IL-10).

As noted previously, the spleens of the vaccinated mice were removed aseptically 14 days after immunization. The single splenic lymphocyte suspensions were prepared and incubated in 96-well plates at 5 × 104 cells/well containing RPMI 1640 plus 10 percent foetal calf serum at 37° in 5 percent CO_2. The cells were stimulated with 50 µl of phytohemagglutinin (50 µg/ml; positive control), 2.5 µg/ml of 146S antigen of FMDV type O/IRN/1/2007 (specific antigen stimulation) and no antigen (negative control), in triplicate. After 48 h, the MTT assay was performed, according to the manufacturer's instructions. The absorbance was measured at 540 nm and the stimulation index (SI) was calculated as the mean OD value of triplicate wells corresponding to cells stimulated with an antigen, divided by the mean OD value of the triplicate wells corresponding to unstimulated cells. The suspension of stimulated splenic lymphocytes after 48 hours was collected for cytokine assay by ELISA (Javan *et al.*, 2020).

Challenge and PD_{50} assay

The treatments resulting in neutralizing antibody titration levels > 1.2 were subject to challenge testing. One hundred fifty guinea pigs (female, weighing about 250 g) were divided into 30 groups of 5 animals each for the viral challenge test. Ten other animals were assigned to a negative control group. For each of the six treatments, groups of five animals each were vaccinated by one of five serial dilutions (1, 1/2, 1/4, 1/8 and 1/16). The vaccination was given subcutaneously, and the booster doses followed at 3-week intervals. On the fourteenth day after the last immunization each guinea pig was

subcutaneously challenged with 0.2 ml of 100 ID_{50} of guinea pig-adapted live virus (seventh passage) injection into the footpad and housed separately for a 7-day period of examination in a BSL-2 laboratory. In the vaccinated animals, total protection was defined as absence of lesions on the footpad; partial protection was defined as lesions occurring on the injected foot only; and without protection was defined when lesions were found on two or more feet and the tongue after challenge. The protective dose50 (PD_{50}) was calculated for the six treatments (Knudsen, Groocock and Andersen, 1979; Rathogwa *et al.*, 2021).

Statistical analysis

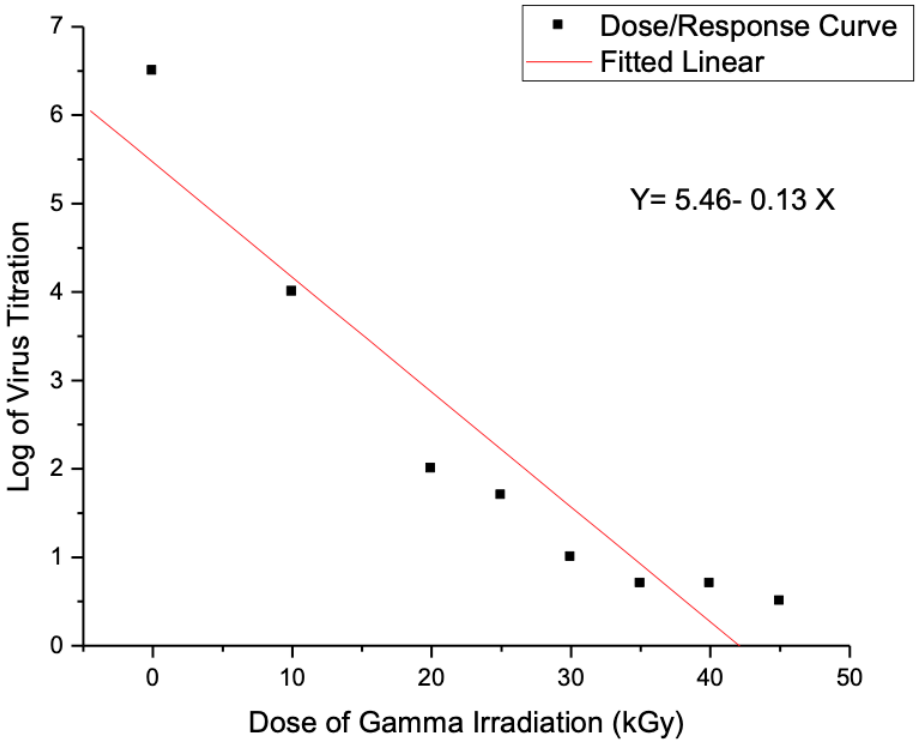
The analysis of variance (one-way ANOVA) followed by LSD test was used for statistical analysis. Differences were considered to be statistically significant when $p < 0.05$.

3. Results

According to the dose/response curves for FMD virus type O/IRN/2010 (Figure 1), with the first titration around $10^{6.32}$ $TCID_{50}$/ml, the D_{10} Value and optimum dose of gamma irradiation for complement inactivation were calculated as 8.33 and 52.5 kGy, respectively. The safety test after four blinded cultures for irradiated virus samples on the BHK_{21} cell line showed that the best dose of gamma radiation for complete inactivation is 50 kGy.

The principal results for immune response to vaccination are in Table 1. The greatest humoral immunity (antibody titration) was observed in the GIV and conventional vaccine groups ($P < 0.05$); the PB1, PB2 and PB3 groups were superior to the negative control ($P < 0.05$). As shown by the SI, cellular immunity was significantly ($P < 0.05$) greater in the PB1, PB2, PB3 and DNA vaccine+GMCSF groups than in the DNA vaccine, conventional vaccine and GIV groups. The SI for all treatment groups were greater than the negative control. The IFN-γ concentration in the GIV group was significantly greater than the negative control ($P < 0.05$), but was significantly less than with the DNA vaccine+GMCSF ($P < 0.05$) and several other options. IL2 and IFN-γ are the cytokines that are secreted from T helper cell class I (Th1). The PB1 and PB2 groups had the highest level of concentration for both of these cytokines ($P < 0.05$). There were no significant differences for IL10 concentration among DNA vaccine, DNA vaccine+GMCSF, PB1, PB2 and the negative control groups ($P > 0.05$), but a significant increase was observed for the other vaccines ($P < 0.05$).

The ID_{50} for guinea pig-adapted live virus (after seventh passages) was calculated as 297 ID_{50}/ml. The quantity was used to challenge of the vaccinated groups for which the antibody titration exceeded 1.2 (i.e. DNA vaccine+GMCSF, GIV, Conventional vaccine, PB1, PB2 and PB3). Table 2 shows the results of the challenge test. The largest PD_{50} were observed for the GIV and PB2.



Source: Author's own elaboration.

Figure 1: The dose/response curves for decreasing of FMD virus type O/IRN/2010 titration against gamma radiation

Table 1: The results of neutralizing antibody titration, splenic lymphocyte proliferation (SI), IFN-γ, IL2, IL4 and IL10 two weeks after second vaccination

No.	Vaccine groups	Antibody titration ±SD	SI ±SD	Mean of IFN-γ concentration (pg/ml) ±SD	Mean of IL2 concentration (pg/ml) ±SD	Mean of IL4 concentration (pg/ml) ±SD	Mean of IL10 concentration (pg/ml) ±SD
1	DNA vaccine	0.9 ± 0^{a}	1.23 ± 0.123^{a}	619.76 ± 144.28^{a}	22.39 ± 8.51^{a}	247.19 ± 72.17^{a}	73.04 ± 52.12^{a}
2	DNA vaccine+GMCSF	1.2 ± 0.17^{a}	1.44 ± 0.131^{b}	$1\,098\pm233.61^{b}$	96.39 ± 25.79^{b}	$1\,339.38\pm1317^{b}$	75.46 ± 67.22^{a}
3	GIV	2.1 ± 0^{c}	1.23 ± 0.046^{a}	386.29 ± 168.45^{c}	74.44 ± 18.34^{b}	$1\,641.80\pm841.64^{b}$	818.78 ± 276.10^{b}
4	Conventional vaccine	2.1 ± 0^{c}	1.26 ± 0.095^{a}	464.27 ± 355^{c}	82.01 ± 32.57^{b}	$1\,278.26\pm1010.56^{b}$	$1\,543.07\pm1171.62^{b}$
5	PB1	1.5 ± 0^{b}	1.52 ± 0.166^{b}	885.76 ± 189.46^{b}	118.66 ± 26.11^{c}	$1\,759.427\pm1153.19^{b}$	16.82 ± 9.48^{a}
6	PB2	1.5 ± 0^{b}	1.55 ± 0.153^{b}	925.76 ± 389^{b}	118.81 ± 42.88^{c}	$1\,988.46\pm1969^{b}$	83.98 ± 31.64^{a}
7	PB3	1.7 ± 0.17^{b}	1.43 ± 0.100^{b}	368.92 ± 82.434^{c}	63.84 ± 5.96^{b}	936.84 ± 329.63^{b}	$1\,698.69\pm1225.94^{b}$
8	Negative control	0.9 ± 0^{a}	0.98 ± 0.051^{c}	3.844 ± 0.58^{d}	1.25 ± 0.39^{d}	79.11 ± 10.18^{c}	32.17 ± 6.87^{a}
9	**Pre-immune**	**0.9 ± 0^{a}**	**0.92 ± 0.07^{c}**	**1.46 ± 0.69^{d}**	**-**	**-**	**-**

Source: Authors' own elaboration.

Table 2: The results of challenge test and PD_{50}

No.	Vaccine groups	Percent of generalization (non-protected animals) in vaccine dilutions					
		1	1/2	1/4	1/8	1/16	PD_{50}
1	DNA vaccine+GMCSF	0	0	40	100	100	4.46
2	GIV	0	0	0	60	100	**7.08**
3	Conventional vaccine	0	0	0	80	100	6.20
4	PB1	0	0	25	57.1	100	6.91
5	PB2	0	0	0	60	100	**7.08**
6	PB3	0	0	16.66	66.66	100	6.28

Source: Authors' own elaboration.

4. Conclusions

Gamma Irradiated FMD virus type O/IRN/2010 can be used for inactivated vaccine preparation. Antibody titration showed that humoral immunity was the same for GIV and conventional vaccine, while it was more than antibody titration in DNA vaccine and DNA vaccine+GMCSF. Also, Cellular immunity in inactivated vaccine groups was less than DNA vaccine+GMCSF. That's why in this research the prime-boost strategy with DNA vaccine+GMCSF and inactivated vaccines (GIV and conventional vaccine) was applied. Furthermore, the increasing of neutralizing antibody response, cellular immunity and Th1 cytokines (IFN-γ and IL-2) caused the prime-boost groups (PB1, PB2 and PB3) were good candidates to make protective immunity against FMD virus. The PD_{50} of GIV and PB2 groups were the greatest. Finally, a heterologous prime boost strategy with DNA vaccine+GMCSF and inactivated vaccine is the best method for inducing humoral and cellular immunity and increasing Th1 cytokines.

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Production and characterization of monoclonal antibodies against a 35 kDa outer membrane protein of ovine *Mannheimia haemolytica*

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Abstract

The role of a 35 kDa outer membrane protein of *Mannheimia haemolytica* in respiratory tract infection in sheep was investigated using monoclonal antibodies (Mabs). Mabs were produced by immunization of BALB/c mice with the detergent insoluble fraction of outer membrane proteins (DIF-OMPs) extracted from ovine field isolate Q11 of *Mannheimia haemolytica*. Five hybridoma producing Mabs were obtained, four of which belonged to subclass IgG_1 and one to IgG_{2b}. SDS-PAGE analysis of whole bacterial lysate and DIF-OMPs extracted from eight ovine field isolates of *M. haemolytica* showed that the 35 kDa appeared as a common band in all isolates, and immunoblot assay showed that all Mabs recognized only a 35 kDa protein. Treatment of OMP antigen with proteinase K or periodic acid showed that the epitope recognized was proteinaceous. Immunofluorescence microscopy on the ovine isolate Q11 reacting directly with Mab Gmh1 conjugated with FITC, or indirectly with unconjugated Mab Gmh1 and goat anti-mouse IgG conjugated with FITC, revealed that Mabs were aggregated intensively on the bacterial surface and capsular material. Indirect ELISA assay revealed that Mabs reacted strongly with all Biotype A isolates of *M. haemolytica* but weakly with several different bacteria including one field ovine serogroup A isolate of *Pasteurella multocida* and the following reference strains: (i) *Clostridium perferingens* strain NCTC 8798; (ii) *Staphylococcus aureus* strain NCTC 10788; (iii) *Salmonella typhimurium* strain NCTC 12023; (iv) *Bacillus cereus* strain NCTC 7464; (v) *Brucella abortus* strain S19; and (vi) *Brucella melitensis* strain Rev-1. The results of the present study showed that by using specific Mabs, the 35 kDa OMP was determined to be surface capsule associated lipoprotein and a highly conserved protein among ovine isolates of *M. haemolytica*.

Keywords

ovine *M. haemolytica*, 35 kDa OMP protein, monoclonal antibody, encapsulation, conserved protein

1. Introduction

Mannheimia haemolytica is a commensal and opportunistic bacterial pathogen that is a causative agent of respiratory tract infection in sheep (Ackermann and Brogden, 2000). The disease, in its typical clinical form, is highly infectious, with a very serious economic impact on the sheep industry worldwide (Donachie, 2007). A number of pathogenic factors of *M. haemolytica*, including the capsule, leukotoxin (LKT), the lipopolysaccharide (LPS), the adhesin, and neuraminidase have been characterized (Highlander, 2001; Mohamed and Abdelsalam, 2008). Several previous studies have suggested that a 35 kDa outer membrane protein (OMP) of *M. haemolytica* plays an important role in immunogenicity and pathogenicity of *M. haemolytica*. It was shown that high antibody responses to several specific OMPs correlated with resistance to challenge with virulent strains of *M. haemolytica* (Confer *et al.*, 1995; Pandher, Murphy and Confer, 1999) and that a 35 kDa heat-modifiable OMP of *M. haemolytica* was involved in adherence to bovine bronchial epithelium *in vitro* (Kisiela and Czuprynski, 2009). Nevertheless, the role of this

protein in the pathogenicity and the mechanism by which the bacteria move from the nasopharynx to the lungs and transfer from commensal organisms to pathogenic bacteria are not yet clarified.

In the Syrian Arab Republic, Pasteurellosis is an officially recognized disease in Awassi sheep, the main breed of sheep in the country. According to the Syrian directorate of animal health, a significant increase in the number of enzootic pleuropneumonia outbreaks has been reported during the last decade. However, little information is available on the role of *M. haemolytica* and its pathogenic factors in respiratory infection in Awassi sheep.

Therefore, and because most reported studies have focused on bovine rather than ovine originated isolates of *M. haemolytica*, the present study was undertaken to characterize a 35 kDa protein of ovine *M. haemolytica* biotype A strains by using monoclonal antibodies.

2. Materials and methods

Bacterial strains and culture media

Nine ovine field isolates, representing eight isolates of *M. haemolytica* (Biotype A) and one isolate of *Pasteurella multocida* (serogroup A) were used in the present study (Table 1). The field isolates were obtained from Syrian Awassi sheep suffering bronchopneumonia (6 isolates) and infected sheep lungs (3 isolates) (Al-haj Ali and Al Balaa, 2019, 2020). In addition, 6 reference strains: (i) *Clostridium perferingens* strain NCTC 8798; (ii) *Staphylococcus aureus* strain NCTC 10788; (iii) *Salmonella typhimurium* strain NCTC 12023; (iv) *Bacillus cereus* strain NCTC 7464; (v) *Brucella abortus* strain S19; and (vi) *Brucella melitensis* strain Rev-1, that were available in our laboratory were also used. The field isolates were grown in brain heart infusion broth (BHI) (Difco, United Kingdom) at 37 °C for 4–6 h and kept in 1 ml aliquots at −80 °C until use. For subsequent experiments, all field isolates were sub-cultured once on BHI agar containing 5 percent sheep blood (BHI-b).

Preparation of killed whole-cell bacterial lysate (Wbl)

Growth conditions for each reference strain are indicated elsewhere. As for the field isolates, an 18 h growth of bacteria on BHI-b was harvested, washed with PBS (pH 7.4) and killed by heating at 100 °C for 1 h (Díaz *et al.*, 2017).

Preparation of detergent insoluble fraction of outer membrane proteins (DIF- OMPs)

DIF-OMPs of *M. haemolytica* were prepared by the method of Bötcher, Lübke and Hellmann (1991). Briefly, harvested bacteria were washed and then re-suspended in 50 mM tris/1 mM Ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0). The bacteria were disrupted by ultrasonication and the supernatant was centrifuged at 40 000 × g for 60 min (Sorvall Discovery 100 SE ultracentrifuge). The pellet was treated twice with N-lauryl-sarcosine-sodium-salt (Sigma-Aldrich, United States of America) in a concentration of 1.5 percent in tris / EDTA buffer. The detergent insoluble fraction was pelleted by centrifugation at 40 000 × g at 4 °C for 60 min and kept in -20 °C until use.

Mice immunization for hybridoma production

Four to six week old female BALB/c mice (Atomic Energy Commission of Syria (AECS) experimental animals farm) were each inoculated twice with 100 µg of OMPs antigen that was extracted from the isolate Q11 (Table 1) and emulsified in Freund's complete or incomplete adjuvants, subcutaneously. One week prior to the fusion, mice were boosted with 125 µl of OMPs only, intraperitoneally.

Table 1: Ovine field isolates of *M. haemolytica* and *P. multocida* that used in the present study

Isolate	Host	Identification	Reference
Q11	Pneumonic sheep[a]	*M. haemolytica*[c]	(Al-haj Ali and Al Balaa, 2019)
Ha2	Pneumonic lamp	*M. haemolytica*	
Ha6	Pneumonic lung[b]	*M. haemolytica*	
Ho8	Pneumonic sheep	*M. haemolytica*	
Ho10	Pneumonic lung	*M. haemolytica*	
Dz14	Pneumonic lamp	*M. haemolytica*	
Rq17	Pneumonic sheep	*M. haemolytica*	
Rq3	Pneumonic lung	*M. haemolytica*	
Ho13	Pneumonic sheep	*P. multocida*[d]	(Al-haj Ali and Al Balaa, 2020)

Sources: a Fisher, M.A., Weiser, G.C., Hunter, D.L. & Ward, A.C. 1999. Use of a polymerase chain reaction method to detect the leukotoxin gene lktA in biogroup and biovariant isolates of Pasteurella haemolytica and P trehalosi. *American Journal of Veterinary Research*, 60(11): 1402–1406.

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Notes: a [a] Bacteria was isolated from nasal swabs of pneumonic Awassi sheep.

b [b] Bacteria was isolated from infected sheep's lung from slaughter house.

c [c] The bacteria was identified on the basis of phenotypic characterization and PCR assay using *Lkt*A primers ([c]) and CAP-A primers ([d]).

Construction of hybridomas

Hybridomas were produced by fusing of plasma cells from immunized BALB/c mice with non- immunoglobulin (Ig)-secreting Sp2/0-Agl4 (SP2/0) myeloma cell line (Zola and Brooks, 1982). Both myeloma and plasma cells were washed, suspended in RPMI growth medium supplemented with 10 or 20 percent of foetal calf serum and tested for their viability. The cells were mixed (1:10), centrifuged and 50 percent of polyethylene glycol 4000 (Sigma-Aldrich, USA) was added as fusion agent. Fused cells were suspended in growth medium containing 1 percent HAT (Gibco, USA), dispensed into 96 well cell culture plates (*TPP*) and incubated at 37 °C in humidified incubator (EuroClone, Italy) in 5 percent CO_2. Those hybridomas producing desired antibodies were cloned three times in HT medium (Gibco, USA) by limited dilution method.

Production and purification of cloned antibodies

Monoclonal antibodies (Mabs) were produced by growing the cloned hybridomas in growth medium and in EX-CELL 610-HSF serum-free medium (Sigma-Aldrich, USA). Antibodies were also produced in pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, USA) primed BALB/c mice and purified by affinity chromatography through affi-gel protein A (MAPII kit; Bio-Rad, USA) as described by the manufacturer.

Indirect ELISA (iELISA)

The hybridoma supernatant was screened by iELISA. Micro plates (*TPP*) were coated with 1 µg / well of Wbl or DIF-OMPs suspension in coating buffer (pH 9.6). After blocking, the hybridoma supernatant along with diluted positive and negative mouse antisera were added to the wells and the plates were incubated at 37 °C for 2 h. The plates were washed and then diluted horseradish peroxidase-conjugated goat anti-mouse IgG (whole molecule, Sigma Aldrich, USA) was added to each well and the plates were incubated at 37 °C for 1 h. The plates were washed and then a substrate consisting of 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.03 percent sodium perborate (Sigma Aldrich, USA) and 0.04 mg/ml of O-phenylenediamine dihydrochloride was added and the absorbance was measured with ELISA reader (Multiskan, Thermo). For titration, OMPs-coated-ELISA-plates were reacted with serial two-fold dilutions of purified Mabs. The end point titre was defined as the highest dilution giving an absorbance 3 times greater than the negative control above background at 492 nm wavelength.

Immunoglobulin subclasses

Immunoglobulin isotyping of Mabs was determined by Mouse monoclonal antibody isotyping test kit (Bio-Rad) as described by the manufacturer.

SDS-PAGE and immunoblot

Wbl or OMPs were electrophoresed and separated with 12.5 percent gel acrylamide. After electrophoresis the samples were transferred to nitrocellulose paper strips. Each strip was blocked and reacted with Mab culture supernatant at room temperature for 2 h. After washing, the strips were reacted with diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) for 1 h. The colour development was achieved by incubation of the strips in a solution of 30 mg 4-chloro-1-naphthol (Sigma-Aldrich, USA) dissolved in 10 ml of methanol and mixed with TBS containing 0.03 percent H_2O_2. The reaction was stopped by washing the nitrocellulose paper in distilled water.

Characterization of the epitope

The epitope recognized by Mabs was subjected for proteinase K treatment or periodate oxidation and tested by iELISA. Proteinase K treatment was performed according to Khosraviani, Nunoya and Matsumoto (1990). Briefly, DIF-OMPs coated plates were washed, and the antigen was exposed to increased concentrations (200–400 µg/ml) of proteinase K in PBS and then incubated at 60 °C for 60 min. Following washing, the Mabs were introduced to the plates and ELISA was performed as described above. Periodate oxidation was performed by the method of Woodward, Young and Bloodgood (1985). Briefly, coated plates were washed with 50 mM of sodium acetate buffer (pH 4.5). Increasing concentrations (30–40 mM) of periodic acid in sodium acetate buffer were introduced to the wells followed by incubation in the dark at room temperature for 1 h. The plates were washed once with sodium acetate buffer and then incubated with 50 mM of sodium borohydride in PBS at 23 °C for 30 min. After this washing, Mabs were added to the wells and ELISA was performed as described above. PBS was employed as control for Mabs.

Conjugation of Mabs with FITC (Mab-FITC)

The Mabs from clone Gmh1 were conjugated with fluorescein isothiocyanate (FITC) using the FluoroTag™ FITC Conjugation Kit (Sigma-Aldrich, USA) as described by the manufacturer. The measurement of the fluorescence intensity was done at 535 nm (excitation at 485 nm) using a fluorescence plate reader (FluoroSkan Ascent FL, Thermo-Electron Corporation, USA).

Immunofluorescence microscopy

For immunofluorescence microscopy, two methods were applied:

Direct labelling: An 18 h growth of *M. haemolytica* strain Q11 was harvested, washed 3 times by centrifugation at 4 000 × g/10 min. in PBS and suspended in 1 ml of D.W. The bacteria (~1.6×10^6) were washed twice with PBS (pH 7.2) containing 0.5 M NaCl, 0.1 percent crystallized bovine serum albumin, 0.05 percent Tween 20 and 5 percent foetal

bovine serum and then were reacted with diluted 1:1500 Mab-FITC at 4 °C with gentle mixing in the dark for 1 h. Labelled bacteria were washed 3 times and suspended in 100 µl PBS containing 2 percent glutaraldehyde.

Indirect labelling: Harvested bacteria were washed, blocked as described above and then were reacted with diluted Mab Gmh1 with gentle mixing. After three washes the bacteria were reacted with 1:1000 diluted goat anti-mouse IgG-FITC antibody (Sigma-Aldrich, USA) at 4 °C in the dark. Labelled bacteria were washed three times and suspended in 100 µl of 2 percent glutaraldehyde in PBS. As a negative control the bacteria of isolate Q11 was stained with DAPI and presented to fluorescence microscopy.

The preparations by the 2 methods above were counterstained with DAPI (40,6-diamino-2-phenylindole) (Abbott Molecular/Vysis, USA) at a final concentration of 2 µg/ml (Al Achkar *et al.*, 2010) and examined with fluorescence microscope (AxioImager.Z1 mot; Zeiss, Germany) equipped with appropriate filter sets to discriminate between a maximum of 5 fluorochromes and the counterstain DAPI. Image capturing and processing were carried out using an image and scanner interface specification (ISIS) imaging system (MetaSystems, Altlussheim, Germany).

Statistical analyses

Each iELISA test was performed in triplicate. Differences in absorbance values were evaluated by one way analysis of variance (ANOVA). All analyses were conducted with version 5.0 GraphPad Prism. *P* values of 0.05 or less were considered statistically significant.

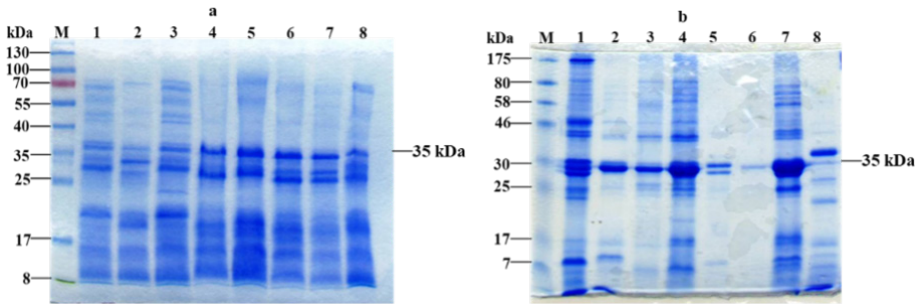
3. Results

Production of Mabs

Based on iELISA and Western blot analysis, a total of 24 hybridomas were obtained and 5 were chosen for further cloning. Hybridomas were cloned three times and stably produced Mabs. The results of Ig isotyping revealed that four hybridomas belonged to IgG_1 and only one Mab to IgG_{2b}. The Mab Gmh1 showed the highest titre, therefore, it was applied in subsequent experiments.

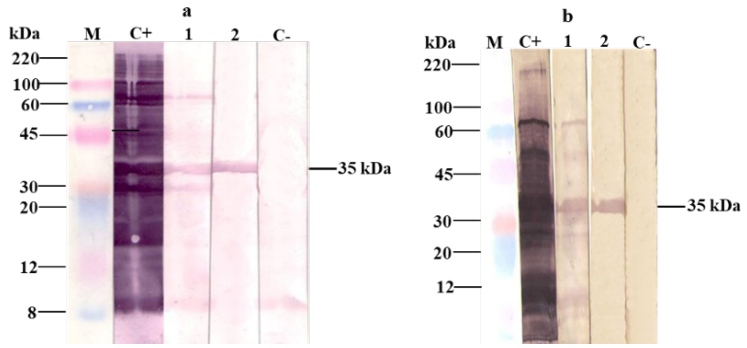
SDS-PAGE and Immunoblot

Protein profiles of Wbl and DIF-OMPs antigens from eight ovine field isolates of *M. haemolytica* are shown in Figure 1a and Figure 1b, respectively. A 35 kDa protein appears as a common protein with various degrees of thickness in all isolates. Reactivity of Mab Gmh1 with DIF-OMPs and Wbl antigens before and after three rounds of cloning is demonstrated in Figure 2. The results revealed that before cloning Mab Gmh1 recognized at least three epitopes at a 70, 35 and 30 kDa positions of DIF-OMPs (Figure 2a lane 1) and Wbl (Figure 2b lane 1), however after 3 successive cloning Gmh1 reacted only with a 35 kDa protein of the isolate Q11 (Figure 2a and Figure 2b, lanes 2).



Source: Author's own elaboration.

Figure 1: Protein profiles of Wbl (a) and DIF-OMPs (b) antigens prepared from 8 field isolates of ovine *M. haemolytica* as shown by SDS-PAGE analysis



Note: M, Low molecular weight marker; Lanes 1-8 field isolates Ha2, Ha6, Ho8, Q11, Ho10, Dz14, Rq17 and Rq3 respectively. 6 µg/lane.
Source: Author's own elaboration.

Figure 2: Immunoblot analysis of Mab Gmh1 reacting with DIF-OMPs (a) and Wbl (b) of *M. haemolytica* ovine field isolate Q11 before (a1 and b1) and after (a2 and b2) 3 times of cloning

Characterization of the epitope

The treatment of DIF-OMPs of the isolate Q11 with proteinase K reduced ELISA values (Figure 3), whereas treatment with periodate had no effect on ELISA means (Data not shown).

Conjugation of Mab with FITC

Fluorescence intensity of Mab-FITC and its ability to bind to a 35 kDa of DIF-OMPs of the isolate Q11 was examined by FluoroSkan. The results showed that Mab-FITC gave significantly higher values of absorbance comparing with unconjugated Mab and that MAB-FITC could tightly react with the epitope at high titres (Data not shown).

Immunofluorescence microscopy

Morphology of the isolate Q11 stained with DAPI is shown in Figure 4a. The bacteria appeared as rods with bipolar staining on the peripheries. The results of the two methods applied for immunofluorescence microscopy on the isolate Q11 are shown in Figure 4b, Figure 4c and Figure 4d. In both methods, Mab Gmh1 appeared to react strongly with the bacteria, which was represented by intense aggregation of FITC-Mab on bacterial surface (Figure 5b and Figure 5c), especially on capsular material (Figure 5d).

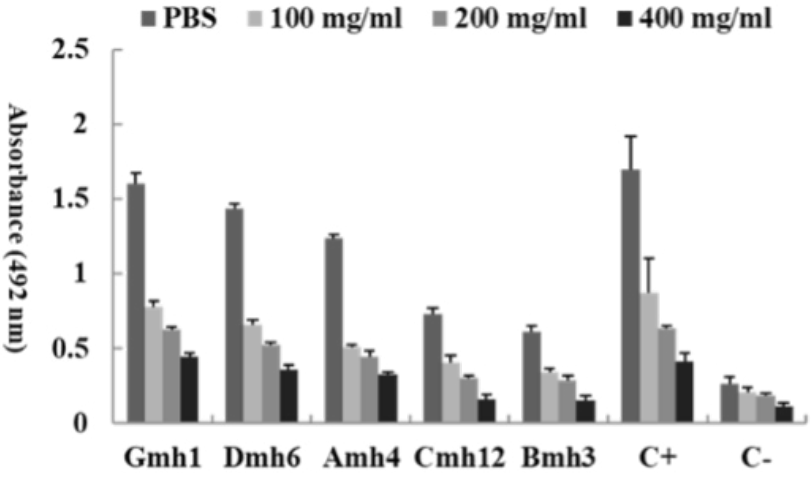
iELISA

Cross-reactivity of Mab Gmh1 with all strains used in the present study was investigated by iELISA. The Mab Gmh1 reacted positively with a 35 kDa protein of all field isolates of *M. haemolytica*, with the highest degree of absorbance being observed for the isolates Q11, Ha2, Ha6, Ho8, and Dz2. On the other hand, a significant decrease in absorbance and very weak reaction were observed with the rest of bacterial strains that were used in the present study including the reference strains and the field isolate Ho13 of *P. multocida*. A statistically significant decrease was observed for two strains: S. aureus NCTC 10788 ($P < 0.05$) and *B. melitensis* Rev-1 ($P < 0.005$), as compared to the reaction of Wbl of the isolate Q11 (Figure 5).

4. Discussion and conclusions

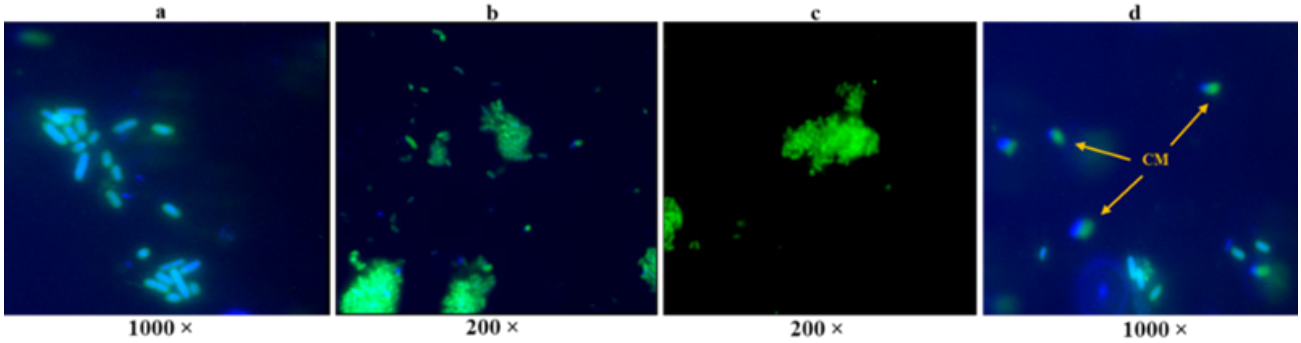
In the present study, five Mabs were generated, and all were directed against the 35 kDa OMP antigen of ovine *M. haemolytica*. Our SDS-PAGE analysis showed that the 35 kDa is a common and major protein for sheep isolates, which indicates that the 35 kDa protein is a major immunogenic antigen for ovine *M. haemolytica*. Mahasreshti *et al.* (1997) identified two major surface exposed outer membrane proteins with molecular weight of 35 kDa (PomA) and 32 kDa (PomB) lipoproteins that were extracted from biotype A of bovine *M. haemolytica*. In their study, cattle vaccinated with live *M. haemolytica* developed a significant increase in serum antibodies to partially purified PomA. Davies and Lee (2004) suggested that the heat-modifiable OmpA is a major structural component of *M. haemolytica*, acts as a ligand, and may be involved in binding to specific host cell receptor molecules in cattle and sheep. Later, Kisiela and Czuprynski (2009) showed that OmpA and lipoprotein 1 (Lpp1) contribute to adherence of *M. haemolytica* to bovine respiratory epithelial cells. Proteinase K treatment of DIF-OMPs reduced ELISA values (Figure 3a) implying that the Mab binding site(s) had been destroyed. However periodic acid, which cleaves neighbouring hydroxyl groups in sugars without altering the structure of the polypeptide chains (Woodward, Young and Bloodgood, 1985), had no effect (Figure 3b), indicating that the epitope recognized by Mabs was proteinaceous. Immunofluorescence microscopy examination showed that the 35 kDa protein was detected at the bacterial surface and cell periphery of *M. haemolytica*. Fluorescent Mabs were not observed on some parts of the bacteria where the capsule was removed, probably during mechanical treatment of the bacteria such as centrifugation (Figure 5d). These results indicate that the 35 kDa OMP is a capsule associated protein. The capsule and the surface proteins of *M. haemolytica* have been implicated in mediating resistance to killing by serum, impairing phagocytosis by bovine neutrophils and alveolar macrophages, and facilitating colony formation in the pneumonic lung of ruminants (Chae *et al.*, 1990; Mohamed and Abdelsalam, 2008).

The results of iELISA with Mab Gmh1 as a primary antibody revealed that Mab reacted strongly with all field isolates of *M. haemolytica* and weakly with other bacterial strains not belonging to *M. haemolytica*, including one field isolate of ovine *P. multocida*. These results combining with the results of SDS-PAGE (Figure 6) indicate that the 35 kDa OMP is a common and highly conserved protein for ovine isolates of *M. haemolytica*. Mahasreshti *et al.* (1997) reported that a 35 kDa protein extracted from *M. haemolytica* is a heat modifiable OMP conserved among *P. haemolytica* biotype A serotypes. The results of the present study confirmed their findings. ELISA assay showed that produced Mabs are specific for *M. haemolytica* only. These results suggest that the Mabs can be applied in development of ELISA based immunoassay for specific detection of sheep infection with *M. haemolytica*. Respiratory infection in sheep is a complicated disease, involving interaction among host (immunological and physiological), multiple agents (bacterial, viral, mycoplasma) and environmental factors. Moreover, *M. haemolytica* is commensal for small and large ruminants. Therefore, it is hard to determine the role of *M. haemolytica* in sheep bronchopneumonia based on classical methods such as bacterial isolation or clinical symptoms only. Thus, applying specific Mabs in iELISA can be useful as an alternative method for diagnosis of sheep Mannheimiosis. More research work is required to clarify this point.



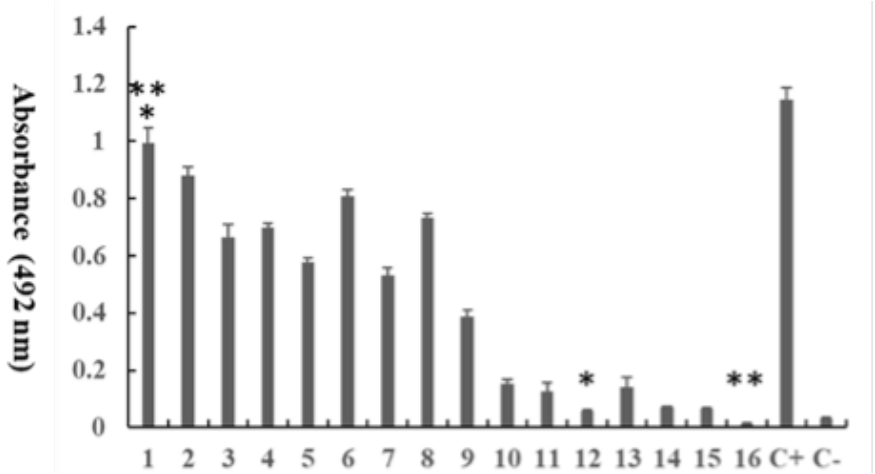
Note: C+, reaction of diluted antiserum from immunized BALB/c mouse with Wbl (aC+) and DIF-OMPs (bC+) of the isolate Q11 (positive control); C-, reaction of diluted serum from unimmunized BALB/c mouse with Wbl (aC-) and DIF-OMPs (bC-) of the isolate Q11 (negative control).
Source: Author's own elaboration.

Figure 3: The effect of treatment of DIF-OMPs antigen from the isolate Q11 with increasing concentrations of proteinase K on the binding of produced Mabs



Note: a, bacteria stained with DAPI; b, bacteria were reacted with Mab Gmh1 and labelled with FITC-conjugated goat anti-mouse IgG; c and d, Bacteria were labelled with Mab-FITC; CM, capsular material.
Source: Author's own elaboration.

Figure 4: Immunofluorescence microscopy of whole bacterial cells of ovine field isolate Q11



Notes: a Diluted Mab Gmh1 was reacted with: 1 and 2 Wbl and DIF-OMP of the isolate Q11; 3-9, *M. haemolytica* field isolates: Ha2, Ha6, Ho8, Ho10, Dz14, Rq17 and Rq3 respectively; 10, *P. multocida* field isolate Ho13; 11-16, reference strains: *C. perferingens* NCTC 8798, *S. aureus* NCTC 10788, *S. typhimurium* NCTC 12023, *B. cereus* NCTC 7464, *B. abortus* S19 and *B. melitensis* Rev-1 respectively; C+, reaction of diluted antiserum from immunized BALB/c mouse with Wbl of the isolate Q11 (positive control); C-, reaction of diluted serum from unimmunized BALB/c mouse with Wbl of the isolate Q11 (negative control).

b *, $P < 0.05$; **, $P < 0.005$.
Source: Author's own elaboration.

Figure 5: Reactivity of Mab Gmh1 with OMPs and Wbl antigens extracted from all strains used in the present study as revealed by iELISA

In conclusion, by production of Mabs, the 35 kDa was determined as a surface capsule associated lipoprotein and is a highly conserved protein among ovine isolates of *M. haemolytica*. By using Mabs, future research work will focus on clarifying the role of the 35 kDa OMP in pathogenicity of ovine *M. haemolytica* on cellular level and development of a specific immunoassay for detection of sheep infection with *M. haemolytica*.

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Development of a multiplex real-time PCR assay for detection of zoonotic abortive agents in livestock

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Abstract

Abortion is one of the major causes of economic losses in the livestock industry. Because several factors can lead to abortion in ruminants, laboratory diagnosis, including molecular detection, of infections causing abortion is often necessary. However, the currently available molecular methods are either time-consuming or costly. Therefore, the purpose of the present study was to develop and evaluate a multiplex assay based on the high-resolution melt (HRM) curve analysis principle for simultaneous detection and differentiation of four zoonotic abortifacient agents in domestic ruminants (cattle, goats and sheep). The existing monoplex real-time polymerase chain reaction (PCR) tests were refined to design an HRM multiplex real-time PCR assay in-silico and tested for *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp., and *Listeria monocytogenes*. The new multiplex PCR assay allowed the highly specific, cost-effective and rapid detection of abortifacient agents based on differences in the melting temperature (Tm) of the PCR amplicons. The assay can be a valuable tool for differential diagnosis of zoonotic abortifacient agents in domestic ruminants.

Keywords

abortive agents, multiplex real-time PCR, pathogen, domestic ruminants, zoonosis

1. Introduction

Abortion is defined as the expulsion of a foetus (dead or alive) of recognizable size at any stage of gestation

(Parleani, 2015). In domestic ruminants (cattle, goats and sheep), abortion has a significant impact on the economies of many countries, primarily affecting animal health and productivity and veterinary public health. Several infectious agents such as viruses, bacteria, protozoa and fungus are accountable for inducing abortions. Non-infectious agents such as stress, housing conditions, transport, toxemia, metabolic or hormonal disorders, nutritional deficiencies, hereditary factors and physical factors can also cause abortion (Beuzón *et al.*, 1997; Vidic *et al.*, 2007; Clothier and Anderson, 2016). Some of the infectious agents such as *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp. and *Listeria monocytogenes* are responsible for abortion in ruminants and also have zoonosis implications (Barkallah *et al.*, 2014).

Diagnosis of abortions in domestic ruminant livestock is complex. Often, such abortions do not present any premonitory signs in affected species, making identifying the aetiological agents difficult. In addition, the numerous causes of abortion make diagnosis quite a challenging and often a frustrating process (Hovingh, 2009). Success rates for abortion diagnoses are low worldwide, with an average ranging between 25 percent to 30 percent in abortion cases submitted to diagnostic laboratories (Holler, 2013). The proper identification of the actual abortion causing mechanism is critical for timely and effective management and control of abortions in ruminants. As *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp. and *Listeria monocytogenes* cause abortion, a rapid differential diagnostic test is needed to facilitate early and accurate detection of these primary zoonotic abortifacients.

Multiplex molecular detection approach provides a good option for differential diagnosis and has the advantages of requiring less input material and allowing higher throughput. In addition, surveillance using the multiplex testing of prevalent pathogens helps in the proper management of animal diseases. Agarose-based multiplex polymerase chain reaction (PCR) assays used over the years to detect pathogens causing abortions (Berri *et al.*, 2009; Tramuta *et al.*, 2011) are time-consuming. On the other hand, multiplex PCR assays using probes have proven highly effective (Reisberg, Selim and Gaede, 2013; Selim, Elhaig and Gaede, 2014; Liu *et al.*, 2016; Sebastiani *et al.*, 2018); however, their implementation is hindered by the expense associated with the probe. The probe is also prone to degradation.

An alternative to using probe-based assays is the use of high-resolution melt (HRM) technology. The HRM involves amplifying the target gene of interest by PCR in the presence of a fluorescent dye and subsequent melting of the amplicons by gradually increasing the temperature. For example, the melting temperature (Tm) of double-stranded DNA is determined by monitoring the loss of fluorescence from a DNA intercalating dye bound to the double-stranded DNA as it denatures into single-stranded DNA at high temperatures (Von Keyserling *et al.*, 2011). The HRM has the advantage of being easy to perform, rapid, flexible and cost-effective.

Multiplex HRM real-time PCR assays have been used to identify, differentiate and genotype many pathogens such as poxviruses (Gelaye *et al.*, 2017), *Mycobacterium tuberculosis* complex (Landolt, Stephan and Scherrer, 2019), *Brucella* spp. (Winchell *et al.*, 2010; Gopaul *et al.*, 2014), *Leptospira* spp. (Naze *et al.*, 2015; Peláez Sánchez *et al.*, 2017), *Listeria* spp. (Jin *et al.*, 2012; Sakaridis *et al.*, 2014), etc. However, there are no multiplex HRM real-time PCR methods available to detect simultaneously in a single tube the potentially zoonotic pathogens commonly responsible for abortions in livestock. Hence, in this study, a single tube multiplex HRM real-time PCR was developed for the simultaneous detection of *Brucella* spp., *Leptospira* spp., *Listeria monocytogenes* and *Coxiella burnetii*, causing abortions in sheep, goats and cattle.

2. Materials and methods

HRM multiplex real-time PCR design and optimization

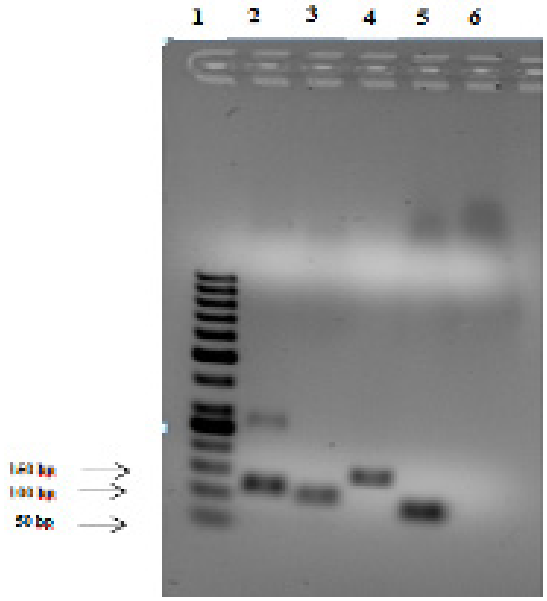
The PCR primers were designed (using Primer 3 software) to target unique sequences in (i) the insertion sequence (IS) element IS711 of *Brucella* spp.; (ii) the outer membrane, surface lipoprotein LiPL32 for *Leptospira* spp.; (iii) the IS1111 gene of *Coxiella burnetii*; and (iv) ssrA gene in *Listeria monocytogenes*. Differences in fragment size and guanine-cytosine (GC) content were used as the criteria for discrimination among species. Accordingly, eight non-labelled primers were produced (Table 1). The total G + C content and fragment size of the predicted PCR amplicons (Table 1 and Figure 1) were calculated using BioEdit software package version 7.1.3.042. In-silico simulation (Figure 1) was performed to design the multiplex HRM assay by using the uMelt software (www.dna.utah.edu/umelt/umelt.html) to predict the Tm of the expected PCR amplicon to avoid overlapping or similar Tm among the pathogens.

The assays were optimized and evaluated in monoplex and multiplex reactions using plasmids harboring the target fragments for each pathogen as positive controls by using the designed primers on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The optimal multiplex HRM qPCR assay conditions consisted of a 20 µL PCR reaction volume containing 1 × SsoFast™ EvaGreen® Supermix (Bio-Rad), 100 nM - 500 nM concentration of each primer and two µL of DNA. The PCR thermal profile was 95 °C for 5 min, followed by 42 cycles of 95 °C for 5 s, 65 °C for 4 s and 70 °C for 5 s. The melting programme was 95 °C for 60 s, cooled to 65 °C for 60 s, and continuous heating from 65 °C to 90 °C at 0.2 °C/ 10 s with fluorescence acquisition.

Table 1: List of the oligonucleotides used in this study

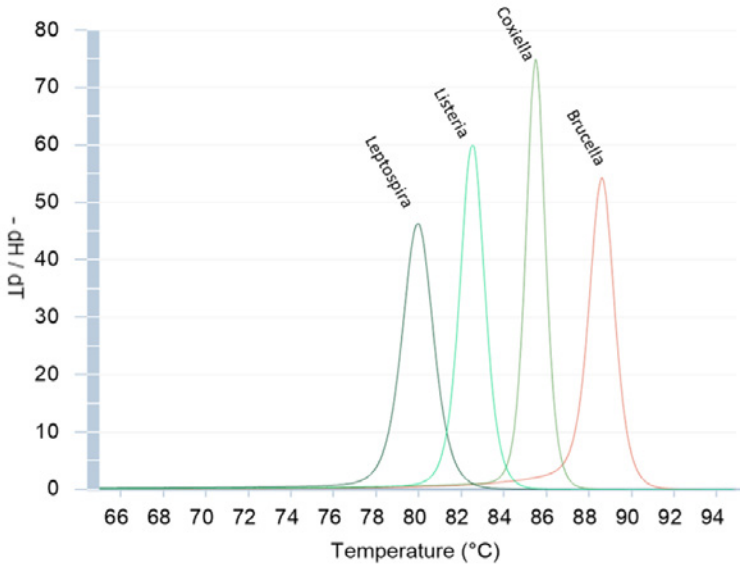
Pathogen	Primer ID	PCR product length (bp)	Total G + C content (%)
Brucella spp.	BruHRM_F BruHRM_R	101	54.08
Coxiella brunetii	CoxHRM_F CoxHRM_R	121	47.05
Leptospira spp.	LepHRM_F LepHRM_R	77	43.22
Listeria monocytogenes	LisHRM_F LisHRM_R	93	42.86

Source: Authors' own elaboration.



Source: Author's own elaboration.

Figure 1: Gel picture showing the expected amplicon size of each of the primers; lane 1: 50 bp ladder; lane 2: *Brucella spp*. (101 bp); lane 3: *Listeria monocytogenes* (93 bp); lane 4: *Coxiella burnetii* (121); lane 5: *Leptospira* spp. (77 bp); lane 6: Negative control without DNA



Source: Author's own elaboration.

Figure 2: Umelt simulation graph showing melting curve, the negative derivative of the fluorescence (F) over temperature (T) (dF/dT) against the temperature (T)

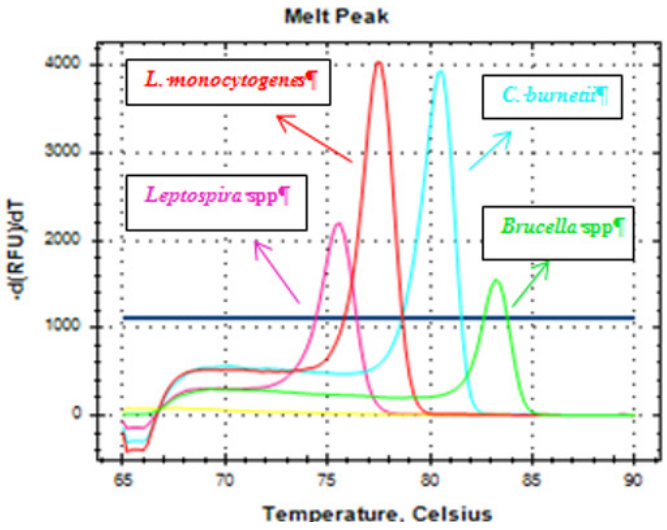
Data analysis was performed by using the CFX Manager™ Software version 3.1 (Bio-Rad, USA), and the amplification and melting curves were displayed as negative first-derivative plots of fluorescence to temperature. High-resolution melting curve analysis was also used to analyze the data, and melting profiles of the four pathogens were determined using the Precision Melt Analysis™ Software version 1.2 (Bio-Rad). Normalized melt curves and differences in curves were acquired by separately analyzing each pathogen's active melt region by designating the corresponding pre-melt (initial fluorescence) and post-melt (final fluorescence) regions.

The HRM multiplex PCR was validated by performing analytical specificity tests and the discriminating power of the assay was determined by using known positive clinical DNA samples from archived samples at Animal Production and Health Laboratory, at least one positive sample for each of the four pathogens. In addition, the specificity of the assay was further evaluated by testing cultured isolates from non-target, closely related bacterial pathogens, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pasteurella multocida*, *Campylobacter* spp., *Trichomonas* spp. and *Salmonella* spp.

3. Results and discussions

HRM multiplex real-time PCR design and optimization

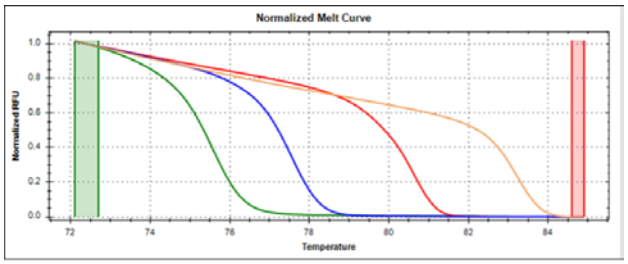
The differences in GC content of the nucleotides allowed the generation of PCR amplicons with a different melting temperature for each of the four targeted pathogens in the study. Thus, the *Brucella* spp. fragment with 101 nucleotides (bp) had a total GC content of 54.08 percent, followed by *Coxiella burnetii* (47.05 percent GC content, 121 bp), *Leptospira* spp. (43.22 percent GC content, 77 bp) and *Listeria monocytogenes* (42.86 percent GC content, 93 bp). As a result, melting peaks (Figure 3) and normalized and difference plots (Figure 4) were produced, enabling the differentiation of the four pathogens. There was no variability observed of Tm peaks of each of the pathogens utilized, in either the monoplex or multiplex assays.



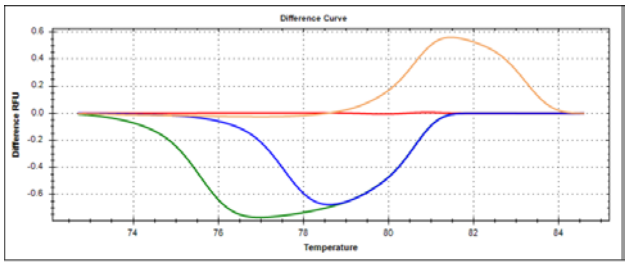
Source: Author's own elaboration.

Figure 3: Melting Peaks of reference plasmids of *Leptospira* spp (75.6 ˚C), *Listeria monocytogenes* (77.4 ˚C), *Coxiella burnetii* (80.6 ˚C) and *Brucella* spp (83.2 ˚C)

A



B



Source: Author's own elaboration.

Figure 4: Normalized HRM plots of the PCR products of four zoonotic abortifacient bacteria; the normalized melt curve and difference curve plots are presented separately with different line colours for each pathogen: Normalized Melt Curve (A) and Difference Curve (B) of *Leptospira* spp., *Listeria monocytogenes*, *Coxiella burnetii*, and *Brucella* spp. Green and red columns in the normalized melt curve plot represent pre- and post- melt normalization regions

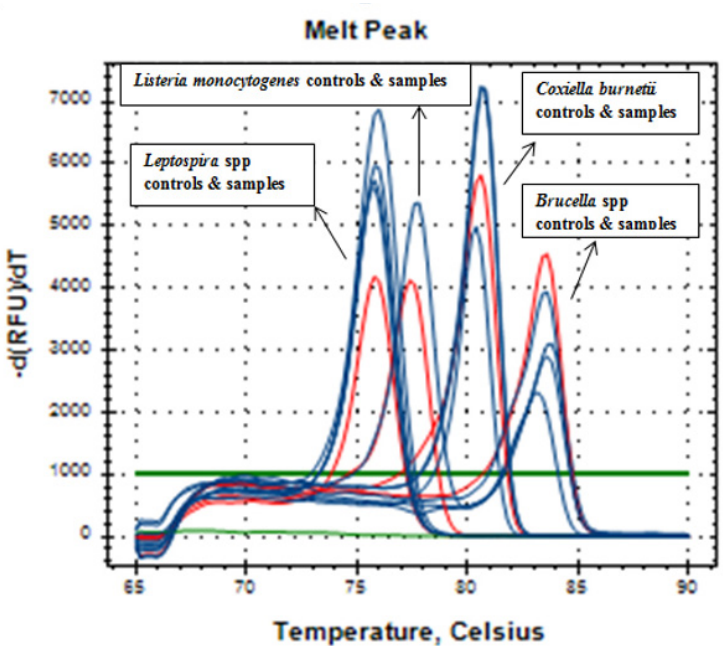
To date, identifying abortifacient agents affecting cattle, goats and sheep is often based on isolation of these pathogens, which is a hazardous, difficult, laborious and time-consuming process (Maurin and Raoult, 1999; Kahn, 2006; Limmathurotsakul *et al.*, 2012; Wolf-Jäckel *et al.*, 2020). Sebastiani *et al.* (2018) reported the development of a multiplex RT-PCR method to identify abortive agents in ruminants; however, this assay is more expensive due to the need for seven different probes. In contrast, the present multiplex HRM real-time PCR does not require a probe or labelled primers, making it easy to set up and interpret the analysis of the melting data.

By simultaneously detecting four abortifacient agents in a single PCR reaction, the developed assay stands as an ideal method to screen abortive zoonotic diseases at a reduced cost, saving time while providing accurate identification of the responsible pathogen. In addition, this will enable early detection of abortifacient agents, enabling proper abortive diseases management, and control at the public health and veterinary level. Ultimately, livestock farmers would receive advice on reducing abortions on their farms.

Validation: specificity and discriminating power of the assay

The positive samples of *Leptospira* spp. (n = 4), *Listeria monocytogenes* (n = 1), *Coxiella burnetii* (n = 3), and *Brucella* spp. (n = 4) were simultaneously detected in a single reaction with clearly differentiated Tm values (Figure 5). This result shows that the assay is very specific, with no inter-species cross-reactivity among the four pathogens. Additionally, the genome of non-targeted pathogens (*E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pasteurella multocida*, *Campylobacter* spp., *Trichomonas* spp., and *Salmonella* spp.) were not detected by the assay.

The preliminary results obtained in this study will undergo further evaluation by testing additional clinical samples to establish it the assay as a routine and efficient surveillance diagnostic tool.



Source: Author's own elaboration.

Figure 5: HRM detection of abortifacient agents; the positive samples (in blue colour) and control (in red colour) of each of the four bacteria displayed a unique melting peak, shown in red colour

4. Conclusions

The developed multiplex HRM assay can simultaneously identify four different bacteria (*Leptospira* spp., *Brucella* spp., *Coxiella burnetii* and *Listeria monocytogenes*) causing abortions in ruminants; it provides a good option for differential diagnosis of zoonotic abortifacient agents. Identification and discrimination of species are based on differences in the Tm of the PCR amplicons obtained after fluorescence melting curve analysis. Therefore, the use of this assay may save time to detect abortifacient bacteria. Additionally, the assay is easy to perform and interpret, a cost-effective and highly specific method that can detect co-infections of the targeted pathogens.

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A snapshot of West Nile virus infections of Portuguese horses between 2016 and 2020

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Abstract

Here we disclose laboratorial data that supports the notion of West Nile virus (WNV) circulation among horses in Portugal during the 5-year period from 2016 to 2020. Blood samples from 165 horses, each showing clinical signs compatible with WNV infection, were analysed. Samples were tested by IgM-ELISA and RT-qPCR for antibody and viral RNA detection. Twenty horses were positive for WNV-specific IgM antibodies, representing a seropositivity of 12 percent. The cases were primarily detected in the south of the country, during late summer and autumn. The laboratorial findings demonstrate the importance of continued surveillance in Portugal given the systematic identification of WNV cases in horses during recent years. The recurrent presence of WNV and its vector in particular geographic areas of the country suggest a legitimate risk of human infection, a topic worthy of attention in a One Health perspective.

Keywords

West Nile virus, zoonoses, horses, Portugal

1. Introduction

West Nile virus (WNV) is a zoonotic mosquito-borne virus of the family Flaviviridae, genus *Flavivirus*, included in the Japanese Encephalitis virus sero-complex. WNV is transmitted in an enzootic cycle between birds and mosquitoes (Kilpatrick *et al.*, 2006; Ferraguti *et al.*, 2016). Mosquitoes become infected after feeding on viraemic wild birds and then transmit the virus to other birds, people and other mammals through their bites.

The WNV has a complex eco-epidemiology that involves a wide range of vectors and enormous host diversity and is considered the most geographically widespread of all mosquito-borne flaviviruses (Ferraguti *et al.*, 2016).

The WNV was first identified in the West Nile district of Uganda in 1937 in a febrile woman, but is nowadays

commonly found in Africa, Europe, North America, the Near East and Asia. In 2018, Europe experienced the largest outbreak ever reported, with the virus spreading across 12 countries in southern and central Europe (Bakonyi and Haussig, 2020). This outbreak and its wide dispersion were attributed to favourable climatic conditions, namely an early spring and very high temperatures during the summer. In European countries, WNV activity is usually detected in late summer and early fall at urban sites near wetlands, where migratory birds and mosquitoes are often congregated at their highest densities (Rappole, Derrickson and Hubálek, 2000).

In humans and horses WNV infections are usually asymptomatic or characterized by a mild febrile illness, although fatal meningoencephalitis or encephalitis may also occur (Paré and Moore, 2018). Approximately 20 percent of horses infected develop clinical signs, which include ataxia (stumbling, staggering, wobbly gait or incoordination) combined with circling, hind limb weakness, inability to stand, multiple limb paralysis, muscle fasciculation, proprioceptive deficits, blindness, lip droop/paralysis, teeth grinding, fever, or acute/sudden death. Thirty percent of the horses that develop neurological disease eventually die, while 10 percent to 20 percent recover with residual neurologic deficits and the remainder recover completely (Paré and Moore, 2018).

Humans and horses are considered "dead-end hosts", given their weak potential to function as amplifying hosts to spread infection onwards. However, due to their susceptibility and close interaction with humans, horses often serve as sentinel species in many countries (Rabinowitz, Scotch and Conti, 2009; Heus *et al.,* 2020). Positive cases indicate that mosquitoes carrying the virus are circulating in the area and warn of the need for mosquito control to reduce the infection potential to other horses and humans.

The first reports of WNV in Portugal date back to the 1960's, with increasing numbers of cases in the last 10 years in horses and birds. Despite this increasing prevalence, only four human cases have been reported to date (Barros *et al.,* 2011; Lourenço *et al.,* 2022).

Immunoglobulin M (IgM) antibody detection by ELISA is the preferred test for WNV diagnosis in live horses, since IgM are short living antibodies and indicate a recent infection (Beck *et al.,* 2017). A positive IgM result, in association with compatible clinical signs, confirms the clinical diagnosis. Detection of viral RNA in field cases is hampered by the typical short duration and low level of the viremia in horses (Beck *et al.,* 2017).

For its importance, West Nile virus is an immediately notifiable disease to the World Organisation for Animal Health (WOAH) through the Animal Disease Notification System (ADNS) (Saiz *et al.*, 2021). Also, at the EU level, WNV infection is notifiable for humans and equids through The European Surveillance System of the European Center for Disease Prevention and Control (ECDC), which publishes weekly WNV epidemiological and geographical distribution updates (Saiz *et al.*, 2021).

In an effort to contribute for the knowledge of the WNV infections in South West Europe, we report here the circulation of the virus among horses in Portugal during the 5-year period from 2016 to 2020.

2. Methods

One hundred sixty-five serum samples used in this investigation had been submitted to the National Institute for Agrarian and Veterinarian Research (INIAV, I.P.) for WNV laboratorial diagnosis, and originated mostly from horses with clinical signs of neurological disease. Equine sera were tested for WNV-IgM specific antibodies by capture ELISA (ID Screen West Nile IgM Capture ELISA, IDVET, Montpellier, France).

Total RNA from blood (n = 165) and from one cerebrospinal fluid (CSF) sample was extracted by using the BioSprint 96 workstation with the MagAttract® 96 cador® pathogen kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). The samples were screened for WNV by real-time quantitative polymerase chain reaction (RT-qPCR) analysis, targeting the NS2A gene (Barros *et al.,* 2013).

3. Results and discussion

Twenty horses were diagnosed as positive, based on the presence of WNV-specific IgM antibodies (Table 1). The total represented a sample positivity of 12 percent (n = 165). None of the horse blood samples tested positive for WNV-RNA, indicating that sampling took place after viremia. This result was not unexpected, since viremia is short-lived in dead-end hosts and already declining substantially once the symptoms begin (Beck *et al.,* 2017). However, one of the seropositive horses tested positive for viral RNA in the CSF sample, despite a very low viral load (Ct = 37.1) (Table 1). This sample was also tested by a generic in-house RT-PCR for sequencing purposes to determine the WNV lineage, but did not yield any results. This may explain the failure of all attempts to isolate the virus, given that the success of virus isolation depends on high viral loads (Saiz *et al.,* 2021).

Although most animals described in Table 1 exhibited clinical manifestations compatible with WNV infection, one IgM seropositive cohabitant did not develop any signs of disease. The most common clinical signs described by veterinary practitioners included ataxia, weakness of limbs, trembling and anxiety. According to the owners, none of these horses had been outside the country, and none was vaccinated against WNV.

Follow-up information was available for only eight horses; three animals had recovered from the disease, while the remaining five died or were euthanized. From these, we were unable to obtain a tissue specimen post-mortem. Among the WNV positive horses, seven were male and thirteen were female.

Except for the last year, in which one positive case was detected in July, WNV infections occurred mainly during October through November. Also, most cases were detected in the south of the country, in predictable regions (Lourenço *et al.,* 2022), close to wetlands and bird sanctuaries.

Table 1: WNV seropositive horses, 2016–2020

Year	Date	Sex	Age	Mortality
2016	August 26	Male	3	Yes
	September 8	Male	NA	NA
	September 14	Male	NA	NA
	October 7	Female	11	No
	October 13	Female	10	NA
	October 24	Female	3	Yes
	November 3	Female	2	NA
	November 8	Male	6	NA
	November 9	Female	10	NA
	November 18	Female	14	Yes
2017	October 3	Female	3	No
	October 10	-	-	-
2018	October 9	Male	26	No
	November 9	Female	2	NA
	November 21	Male	12	NA
2019	October 14	Female	17	Yes
	October 26	Female	10	NA
	October 27	Male	11	NA
2020	July 24	Female	1	Yes
	September 29	Female	10	NA

Source: Authors' own elaboration.
Note: NA- data not available.

In a recent study conducted by our team, WNV neutralizing antibodies were also detected in ten (13.70 percent) out of 73 wild bird samples collected in 2019, corroborating the circulation of the virus in the country (Costa, 2021).

In 2016, the number of cases detected in horses in Portugal was higher ($n = 10$) than in any other year. Since then, and until 2020, the number of annual cases remained stable (namely $n = 2$, $n = 3$, $n = 3$ and $n = 2$), reflecting the continued presence of the virus.

Due to the close geographic proximity to Spain and the time frame of appearance of the Portuguese cases of WNV along with Spanish outbreaks strongly support the same origin of viruses. In the 2016 season, Spain reported, for the first time, three human cases of West Nile Neuroinvasive Disease (WNND) and 68 horse cases in the Andalusia region, close to the Portuguese border (López-Ruiz *et al.,* 2018).

There are two main WNV genetic lineages. Lineage 1 is responsible for the majority of the outbreaks in horses and humans in Europe, Africa, the Near East, India, Australia, and North America (Bakonyi *et al.,* 2006). Lineage 2 is believed to have entered Europe two years before its initial isolation in Hungary in 2004, and could have occurred through migratory birds that were infected in Africa and remained viraemic during migration, allowing them to infect mosquitoes in Europe upon arrival (Bakonyi *et al.,* 2006; Bakonyi *et al.,* 2013; Hernández-Triana *et al.,* 2014). The genetic characterization of Lineage 2 strains detected in Austria (2008), Greece (2010), Italy (2011, 2013) and Serbia (2012), strongly suggest that the spread of Lineage 2 throughout Europe was due to spread of the 2004 Hungarian strain, rather than from separate incursions via other migratory birds from Africa (Bakonyi *et al.,* 2006).

The lack of viral RNA detection so far has hampered the genetic characterization of the strains that circulates in Portugal, and consequently the molecular epidemiology analyses. As such, it is not clear if residually endemic transmission of WNV occurs continuously in Portugal, or if the sporadic virus circulation following new seasonal introductions from migratory birds takes place every year.

In Portugal, most of the surveillance remains passive, responding to occasional reports of clinically WNV-compatible events in horses and humans. Since infections

tend to be asymptomatic or mild in both species, it is highly probable that only a very small percentage of all infections end up being diagnosed.

A recent work (Lourenço *et al.*, 2022) reviewed all existing data regarding WNV in Portugal from 1966 to 2020, and used climatic data to evaluate, for the first time, WNV transmission suitability using computational approaches over the period 1981–2019. That study reported that although WNV-vector mosquitoes are spread over the entire country, molecular and serological WNV evidence is vastly concentrated in the south where the only four human cases have been reported to date. Accordingly, the computational estimations of WNV suitability found quantitative differences between the north and south of Portugal, suggesting the south to be more suitable for transmission. The model predictions agree with the clinical and laboratorial data, which show that WNV circulates mostly in the south of Portugal, infecting birds, horses and, in rare instances, humans. That study also showed that long-term trends in climate change are making Portugal slowly more suitable for successful WNV transmission (Lourenço *et al.*, 2022).

The profound environmental and climate changes that have been affecting the natural ecosystems are considered major causes for the emergence of new infectious diseases in animals and humans (El-Sayed and Kamel, 2020). In this scenario of emergence and re-emergence of pathogens, sentinel species for zoonotic infectious diseases, such as horses for WNV infection, are a key concept that should be embraced and explored by animal, human and environmental professionals. This holistic approach has the potential to gather and provide important integrated epidemiological information that can save human and animal lives. In fact, surveillance of WNV based on sentinel horses have been triggered in Spain (Jiménez-Clavero *et al.*, 2010), Germany (Ziegler *et al.*, 2013) and Morocco (Benjelloun *et al.*, 2017).

4. Conclusions

This study contributes to increase the knowledge about WNV circulation in southern Europe. The results indicate continual circulation of WNV in Portugal from 2016 to 2020, and demonstrate the importance for continued and targeted WNV surveillance in the country. The repeated identification of WNV cases in horses over the last five years clearly indicates the recurrent presence of a viral pathogen and its vector, in particular, southern geographic areas of the country, disclosing the real risk of infection for humans.

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Diagnosis of *Cryptosporidium* spp. in neonatal calves by ELISA, nested PCR and carbol fuchsin staining methods

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Abstract

Cryptosporidiosis is a common diarrhea-inducing coccidian parasitic infection in animals found throughout the world, that is frequently afflicting immunosuppressive adults and children. Many techniques have been developed for detection of *Cryptosporidium* oocysts. The objective of this study is to diagnose cryptosporidiosis in neonatal calves by carbol fuchsin staining, enzyme linked ımmunosorbent assay (ELISA) and nested PCR and to compare these methods. A total of 150 faecal samples were collected from calves (≤ 2 months) with diarrhea. Fifty-two (34.6 percent) faecal samples were found to be positive by the carbol fuchsin staining method and 56 (37.3 percent) gave a positive reaction for *Cryptosporidium* spp. with ELISA (ELISA-Sandwich). The nested PCR of 150 fecal samples showed a total of 56 (37.3 percent) positive amplifications. This study indicates that the carbol fuchsin staining method can be used for diagnosis of clinical cases, however nested PCR and ELISA are more sensitive than conventional staining methods.

Keywords

Cryptosporidiosis, carbol fuchsin, ELISA, nested PCR, calves

1. Introduction

Cryptosporidium is a widely distributed coccidian parasite that causes enteric disease in humans and animals. It has been reported as a common cause of diarrhoea outbreaks in newborn and young calves (Fayer, 2004; Ebrahimzadeh *et al.*, 2009). *Cryptosporidium* has gained much attention in the last 20 years as a clinically important human pathogen. Presently, the increasing population of immunocompromised people and the various outbreaks of cryptosporidiosis through infection by waterborne *Cryptosporidium* oocysts have placed an even greater emphasis on control of this pathogen (Hannahs and College, 2022).

In cattle, cryptosporidiosis is mainly a problem in neonatal calves. The parasite frequently acts alone, but the losses are pronounced when concurrent enteropathogens are present. Economic losses associated with cryptosporidiosis are due to retarded growth and mortality, and it is difficult to estimate costs resulting from interventions necessitated by diarrhoeic problems (de Graaf *et al.*, 1999).

Early diagnosis of *Cryptosporidium* spp. is central to the control of this disease. There are a variety of methods used by laboratories around the world, including microscopy, immunological and molecular techniques for the detection of *Cryptosporidium* oocysts. Microscopic methods include concentration techniques and staining of faecal samples (e.g. with carbol fuchsin). It has been reported that the enzyme linked ımmunosorbent assay (ELISA) for *Cryptosporidium* can detect the pathogen in a greater proportion of specimens than microscopic examination, and is sufficiently sensitive and specific to detect clinical cases of cryptosporidiosis (Sevinc, Irmak and Sevinc, 2003). In epidemiological screenings and genetical examinations, utilization of the polymerase chain reaction (PCR) with its high sensitivity and specificity is highly beneficial (Carey, Lee and Trevors, 2004; Sakarya *et al.*, 2009).

The purpose of this study is to compare carbol fuchsin staining method, ELISA and nested PCR used in routine examination of samples for *Cryptosporidium* spp.

2. Material and methods

One hundred and fifty faecal samples were collected from calves (≤ 2 months) with diarrhea. Samples were kept at +4 ℃ until laboratory analyses. To reveal the presence of *Cryptosporidium* in the faeces, microscopy via the carbol fuchsin staining method, ELISA and nested PCR were employed. The carbol fuchsin staining method was performed according to the methods described by Heine (Heine, 2010).

A commercial ELISA kit (Diagnostic Automation, Inc., USA) was used for detection of *Cryptosporidium* copro-antigen in this study. It is a double antibody (sandwich) ELISA using an anti-*Cryptosporidium* antibody to capture the antigen from the stool supernatant. A second anti-*Cryptosporidium* antibody is then added, which "sandwiches" the captured antigen. Samples were processed according to the manufacturer's recommendations and results were assessed at a wavelength of 450 nm using an ELISA reader. An absorbance reading of ≥ 0.15 optical density (OD) units was interpreted as an indication that the sample contained *Cryptosporidium* antigen.

After homogenizing, a 200 µl aliquot was taken from each faecal sample and DNA extraction was performed using the QIAamp DNA Stool mini kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. DNAs were stored at -20 ℃ until used in PCR. *Cryptosporidium* specific primers were used to amplify a fragment of the 18S rRNA gene by Nested PCR as described by Xiao *et al.* (2001). The 18S rRNA genes are commonly chosen as a target region for the *Cryptosporidium* genus. The sensitivity and specificity of these primers have been confirmed in previous studies (Xiao *et al.*, 1999; Xiao *et al.*, 2001).

Briefly, for the first stage of the PCR, a PCR product of about 1325 bp was amplified with primers: 5'-TTC TAG AGC TAA TAC ATG CG-3' (forward) and 5'-CCC TAA TCC TTC GAA ACA GGA-3' (reverse). As the template 1 µl DNA was used. The secondary PCR product of 826-864 bp was then amplified from 2 µl of the primary PCR product with primers 5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3'and 5'- AAG GAG TAA GGA ACA ACC TCC A-3'. The PCR mixture contained 1X PCR buffer, 6 mM MgCl2, 200 mM (each) deoxynucleoside triphosphate,100 nM (each) primer, 2.0 U of HotStartTaq DNA polymerase (Fermentas, USA), and 1 µl of DNA template in a total 25 µl reaction mixture.

Each PCR cycle consisted of 35 cycles of denaturation at 94 °C for 45 sec, annealing at 57 °C for 45 sec, and extension at 72 °C for 1 min; an initial denaturation step consisting of incubation at 94 °C for 3 min and a final extension step consisting of incubation at 72 °C for 7 min were also included. The PCR mixture and cycling conditions were identical to the conditions used for the primary PCR step, except that 3 mM MgCl2 was used in the PCR mixture. Control samples were run in each PCR. Sterile water (DNase, RNase free) and *Cryptosporidium* spp. positive control DNA were used as negative and positive controls in PCR, respectively. *Cryptosporidium* spp. positive control DNA was provided from the Department of Parasitology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University in Efeler/Aydın, Türkiye. These samples had been previously identified as *Cryptosporidium* spp. by nested PCR (Aysul *et al.*, 2009). The PCR products were analysed by 1.5 percent agarose gel electrophoresis after ethidium bromide staining.

3. Results

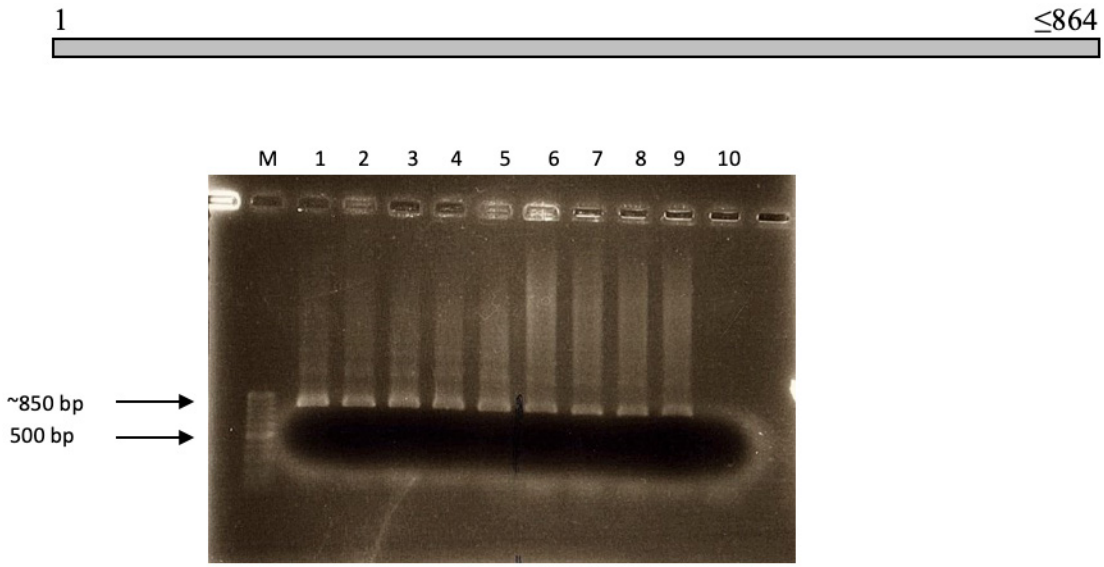
The results of the experiment are presented in Table 1. In this study, 52 (34.6 percent) of 150 samples examined by the carbol fuchsin staining method were found to be positive, based on observation of *Cryptosporidium* spp. oocysts. Fifty-six (37.3 percent) of the samples gave positive reactions for *Cryptosporidium* spp. with ELISA. The nested PCR also showed 56 (37.3 percent) positive amplifications in 150 faecal samples.

All the carbol fuchsin positive specimens were detected as positive by ELISA and nested PCR methods. All samples that were positive by ELISA were also detected as positive by nested PCR. An example of nested PCR scanning results of positive faecal samples is shown in Figure 1.

Table 1: Positive results of carbol fuchsin staining, elisa and nested pcr methods (N = 150)

Method	No. of positives	Positive rate %
Carbol fuchsin staining	52	34.6%
ELISA	56	37.3%
Nested PCR	56	37.3%

Source: Authors' own elaboration.



Source: Author's own elaboration.

Figure 1: Nested PCR scanning results of representative positive faecal samples; *Cryptosporidium* spp. The 826-864 bp region of the 18S rRNA gene was duplicated M: 1 kb marker, 1-8 Positive samples, 9: Positive control 10: Negative control

4. Discussion and conclusions

Direct microscopy, immunological and molecular techniques are routinely employed for detection of *Cryptosporidium* oocysts in faeces in both human and calves (Sevinc, Irmak and Sevinc, 2003; Sakarya *et al.*, 2009; Simsek *et al.*, 2012; Güven *et al.*, 2013; Bhat *et al.*, 2014). The prevalence of cryptosporidiosis in Türkiye varies between 7.2–63.9 percent in calves (Aysul *et al.*, 2009; Sakarya *et al.*, 2009; Simsek *et al.*, 2012; Güven *et al.*, 2013). A previous study was performed by Kabir *et al.* (2020) to analyse the detection of *Cryptosporidium* genotypes and subtypes from young ruminants in Türkiye. The results indicated the high infection of *Cryptosporidium* and proposed that young ruminants are likely a major reservoir of *C. parvum* and a potential source of zoonotic transmission (Kabir *et al.*, 2020).

Danišová *et al.* (2018) compared three commercially available immunological tests and they used PCR to confirm their results. The reported prevalence of cryptosporidiosis in 79 diarrheic animals by enzyme immunoassay, ELISA, immuno-chromatographic test and polymerase chain reaction were 34.17 percent, 27.84 percent, 6.33 percent and 27.84 percent, respectively. They stated the use of various *Cryptosporidium* species in diagnosis based on immunological testing and different results obtained by individual tests potentially indicated differences in the Copro-antigens produced by the individual *Cryptosporidium* species. Our positive results using ELISA and molecular methods were similar to those reported by Danišová *et al.* (2018).

In another research study, Mahmoudi *et al.* (2021) compared diagnosis of *Cryptosporidium* in stool samples of calves by acid-fast staining, ELISA and PCR methods. They indicated that PCR and ELISA were more accurate than the microscopic method. The current study results were in line with those recorded by Mahmoudi *et al.* (2021).

Direct demonstration of the oocysts in the faeces is considered to be the gold standard in conventional parasitology, and has for years been the customary practice for the diagnosis of gastrointestinal protozoal disease (Paul *et al.*, 2009; Bhat *et al.*, 2014). The carbol fuchsin staining technique has been found to be laboratories to be advantageous due to its convenience and practicability, low cost and ability to provide quick results. On the other hand, staining techniques require an experienced microscopist, and the sensitivity and specificity may vary when the shedding of oocysts is intermittent or in low concentration. In fact, the results of this study found that both PCR and ELISA were more sensitive and specific than the conventional staining used. According to our findings, it is suggested to apply ELISA for determination of *Cryptosporidium* spp. in veterinary diagnostic laboratories. ELISA is a reliable and relatively inexpensive technique if the necessary equipment is available. Meanwhile, the PCR method is a costly technique, and it is more appropriate for detailed studies and researches.

In conclusion, new and rapid diagnostic techniques such as ELISA and PCR have played an important role in animal health management and control of cryptosporidiosis. These techniques are easy to use, rapid, sensitive, specific and can offer significant advantages over traditional staining methods. Fast action can not only limit damage to the affected herds, but also prevent the diseases from spreading into neighbouring herds and villages or even other countries.

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Fascioliasis and the metabolomic study of its effects on livestock productivity

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Abstract

Diseases caused by parasites decrease the health and fitness of many animals and the condition is particularly concerning in livestock because of their great economic importance throughout the world. Among the parasitic diseases, Fascioliasis caused by *Fasciola hepatica*, is the most important parasitic disease affecting ruminants in the subtropical and tropical regions of Mexico. The prevalence is estimated between 23.5 to 33 percent in very humid areas with temperatures that vary from 15.5 ± 1.1 to 34.1 ± 2.1 °C and at altitudes that range between 300 and 2 400 masl. The objective of this study was to determine the pathophysiological changes (histological analysis, numbers of flukes in liver parenchyma, metabolic and enzymatic profiles, and differential counts of blood cells) caused by *F. hepatica* in native Creole cattle in different zoogeographic areas in Mexico. Blood and liver tissue samples were taken from 7 892 animals. The prevalence of fascioliasis in native cattle in this study was found to be 48.9 percent. Macro histopathological examination of the samples revealed liver obstructions, an increase in the diameter and thickening of the bile ducts (cholangiohepatitis), and a decrease in the thickness of the left and ventral liver lobes. Liver cirrhosis was observed with livers of up to 30 mm thick. There was loss of cellular morphology and presence of *F. hepatica* eggs, which were deposited in the liver parenchyma and in the blood and bile ducts. Liver damage was found to be directly proportional to the number of flukes found and the flukes also caused alteration in blood biochemical components. The biochemical parameters in blood were very significantly altered ($P < 0.05$). These altered parameters were the following: urea, cholesterol, total protein, and enzymes including gamma glutamyl transferase, alanine amino-transferase; aspartate amino-transferase, lactic dehydrogenase and alkaline phosphatase. These effects were observed in both female and male animals and varied generally according to the number of flukes present in the liver. In conclusion, fascioliasis constitutes one of the most important parasitic diseases in many regions of Mexico because of poor diagnosis, wide distribution of intermediate hosts, and inadequate prevention and control measures. A high prevalence of transhumance contributes to the spread of this parasitic disease of global importance in Mexico.

Keywords

Fasciola hepatica, pathophysiology, liver damage, parenchyma, biochemical parameters

1. Introduction

Mexico is one of the countries in Latin America with the highest number of ruminants (cattle and sheep). The ruminant population is affected by a parasitic disease called fasciolosis, which affects herbivorous animals and, less frequently, humans. This parasitosis is caused by a parasite of the Trematoda class, family Fasciolidae: *Fasciola hepatica* (Dalton, 1999). Physiological damage from this parasitic disease causes heavy economic losses in livestock, by causing a negative effect on weight gain (Loyacano *et al.*, 2002), reproductive efficiency and milk production (Chirinos and Martínez de Chirinos, 1993) and the confiscation of livers from infected animals (Ortega *et al.*, 2007).

This disease is emerging or re-emerging in many countries, and its prevalence, intensity, and geographic distribution are increasing (Mas-Coma, 2004). Fascioliasis is currently the vector-borne disease with the widest known

latitudinal, longitudinal and altitudinal distribution (Mas-Coma *et al.*, 2003). This parasitosis affects many regions of Mexico, with a prevalence of 33 percent in cattle in very humid areas (tropical and subtropical), 13 percent in sheep, and 65 percent in buffaloes. Although the prevalence of this parasitosis had decreased in the past 10 years in those regions where feed additives such as beta-adrenergic agonists (β_2-AA) were administered, there has recently been increase in liver tumours and, possibly, these same additives disguise infestations of *F. hepatica* or accelerates liver damage, which may decrease the defensive capacity of the liver parenchyma. Fascioliasis has been detected in animals with high levels of serum concentrations of β_2-AA (Clenbuterol-Clb) - 1253.5 ± 87.5 to 1850 ± 123.4 ng/ml (Caicedo *et al.*, 2009; Paz-Calderón Nieto, Caicedo Rivas and Hernández Pérez, 2011; Caicedo, Paz-Calderón Nieto and Badillo, 2011; Saavedra Rodríguez *et al*, 2019). Recently, diagnostic and control techniques have been implemented for this parasitosis, but these programmes have mostly been established by higher education institutes and have not encouraged government authorities to allocate more resources for further study and expanded application. Thus, the current extent of control measures for fascioliasis do not match its scope and repercussions in terms of damage to the national economy. The purpose of this study was to evaluate the pathophysiological effects caused by *F. hepatica* in native Creole cattle in different zoogeographic zones of Mexico.

2. Materials and methods

Blood and liver samples

Samples were collected from 7 892 clinically healthy cattle, slaughtered in different municipal slaughterhouses and private farms in Mexico, for five years.

Histological analysis

The number of trematodes in the liver parenchyma was determined by examining the affected livers. To observe the damage of liver parenchyma, liver tissue samples were taken and subjected to a histological process and stained with H&E (hematoxylin and eosin).

Blood samples

Blood was taken to count white blood cells (differential count). Traceability of samples was addresses by assigning a code to each sample and matching it to an existing animal identification number. The analysis of the metabolic profile was carried out in blood serum samples, macro minerals were measured as; calcium (Ca) and phosphorus (P), metabolites including blood urea nitrogen (BUN), cholesterol, total proteins (TP), bilirubin, and the enzymes gamma glutamyl transferase (γ-GT), alanine amino-transferase (ALT); aspartate amino-transferase (AST), lactic dehydrogenase (LDH), and alkaline phosphatase (ALP) were detected with different diagnostic kits (Bio-System-USA).

Statistical analysis

An analysis of variance (ANOVA) with the statistical program Stat-2 was used (Olivares, 1994) to test the significance of potentially influential factors and Duncan's new multiple range test was used to determine the significance between the averages.

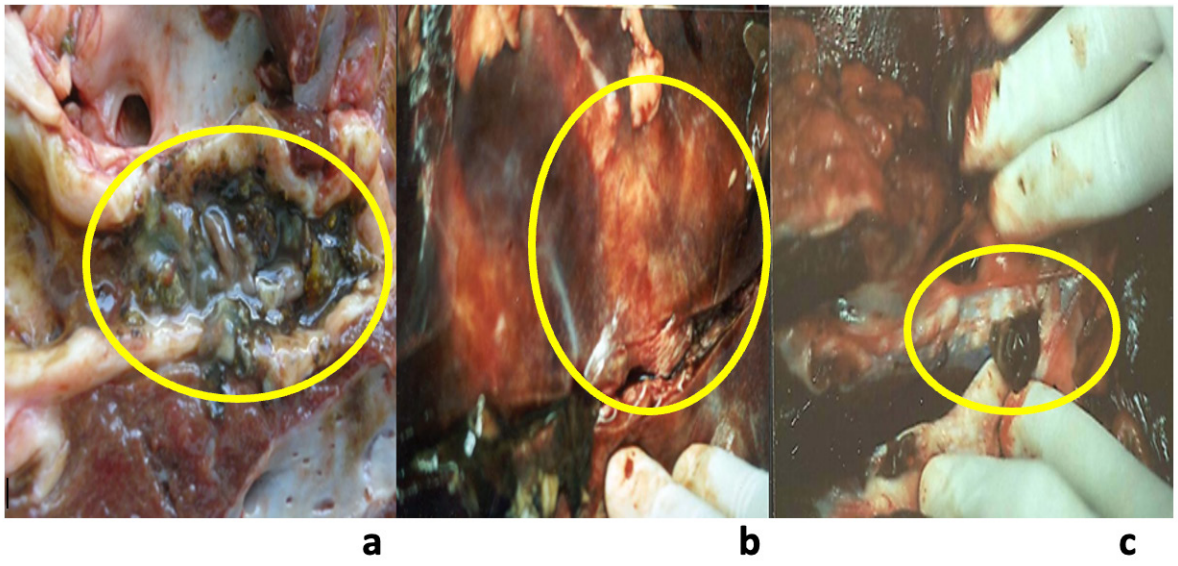
3. Results and discussion

Number of flukes in liver parenchyma

Among the 7 892 animals initially sampled, 1 870 cows and 1 988 males were selected at random for further study. Among these animals, 914 cows and 996 bulls were positive for the presence of *F. hepatica*. Flukes were found in a variable number in the liver ducts of affected animals, with a minimum of 1 to a maximum of 273. The largest number of flukes located in the liver parenchyma of any sampled cow was 54, while in bulls the maximum was 273. The variation in the number of flukes detected in the liver parenchyma will depend on the number of metacercariae ingested by each animal, which was impossible to know from this study, because the animals analysed were infested was naturally by *F. hepatica.*

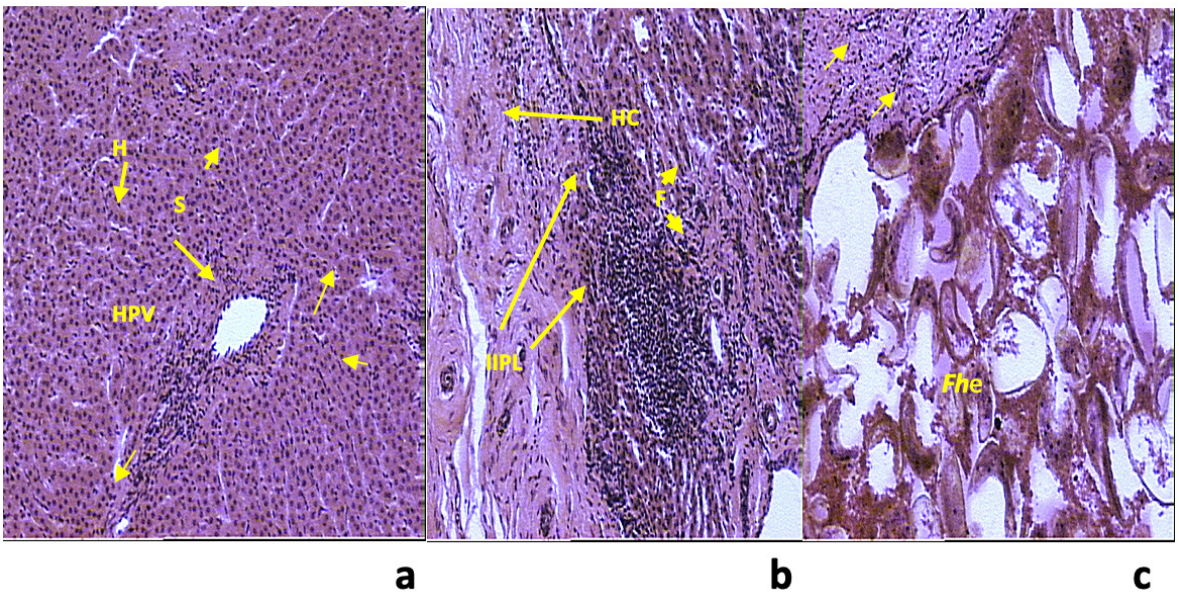
Consistent with observations by Dalton (1999), most of the sampled livers showed an increase of about 3 cm in diameter of the liver ducts. The colour of affected ducts was whitish-yellowish, due to the obstruction of these ducts by the parasite (Figure 1a). Decreases in the thickness of the affected lobes (left and ventral) as the disease progressed were observed, as was loss of liver consistency, due to fibrosis and cirrhosis in chronic cases (Figure 1b). As a result, the liver loses its metabolic faculties and allows the accumulation of substances rich in salt, which then crystallizes, causing obstruction of ducts (Figure 1c). This results in favourable conditions for the reproduction of bacteria such as *Clostridium spp* (Robles, 1998), *Escherichia coli, Enterococcus faecalis,* y *Klebsiella pneumoniae* (Valero *et al.*, 2006). Comparing the above characteristics with the descriptions of disease progression in the literature (Neff *et al.*, 2001; Mas-Coma, 2005) indicates that most of the animals with *F. hepatica* in this study were in the chronic phase of the disease.

At the cellular level, comparing with clinically healthy liver tissue (Figure 2a), we observed loss of the morphology, chronic hepatitis, inflammation forming micro-abscesses, proliferation of bile ducts, intracytoplasmic cholestasis, chronic inflammatory infiltrate based of lymphocytes, dilation of the sinusoids (Figure 2b), thickening of the capsule of Glisson by a chronic process, presence of fibroblasts, fibrosis, cirrhosis, and eggs of *F. hepatica* in the ducts and in the hepatic parenchyma (Figure 2c).



Source: Author's own elaboration.

Figure 1: a) Opening of a hepatic duct where crystallized bile and flukes of *F. hepatica* are observed; b) change of the liver coloration (fibrosis-cirrhosis); and c) crystallization of bile fluid within liver ducts, this is cholangiohepatitis



Source: Author's own elaboration.

Figure 2: (a) Section of clinically healthy bovine liver, showing hepatocytes (H), sinusoidal ducts (S), bile ducts (BD), hepatic portal vein (HPV), terminal hepatic vein (THV); (b) Section of the Creole bovine liver with fascioliasis, showing the presence of an inflammatory infiltrate with a predominance of lymphocytes (IIPL), proliferation of capillaries, fibrosis (F), hepatocyte cords with degenerative changes (HC); replacement of the liver parenchyma by fibrosis and thickening of the capillary wall with an inflammatory infiltrate; (c) Accumulation of *F. hepatica* (Fhe) eggs surrounded by tissue necrosis (NT) and cellular detritus, delimited by fibrous liver tissue (FT) with an inflammatory infiltrate

Differential recount

Regarding the counting of white blood cells, we found significant differences ($P < 0.05$) in eosinophil values in the healthy versus infected groups, as well as in monocytes in males (Table 1). When infected animals were grouped according to the number of flukes (Table 2), groups of males differed significantly. Severely infected males (> 51 flukes) had a decrease of neutrophils and monocytes, and an increase of lymphocytes and eosinophils relative to healthy animals, whereas males with fewer flukes tended to have intermediate counts that only differed significantly from healthy animals in a few instances. The group of females with the largest number of flukes had decreased monocytes and an increase of eosinophils.

The increase in the number of eosinophils in animals with *F. hepatica* concords with the diagnostic characteristics of the disease (Neff *et al.*, 2001; Mas-Coma, 2005); however, this increase is more evident during the parenchymal phase and these cells increase when the parasite enters the bile ducts (Poitou, Baeza and Boulard, 1993; Jemli *et al.*, 1993).

Table 1: White blood cell counts of clinically healthy bovines and bovines with *fasciola hepatica*

		n	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)	Basophils (%)
Males	Healthy	992	54.9 ± 1.6	38.7 ± 1.5	2.0 ± 0.3[a]	3.8 ± 0.4[a]	0.6 ± 0.1
	F. hepatica	996	47.8 ± 3.0	45.7 ± 2.9	4.3 ± 1.1[b]	1.8 ± 0.4[b]	0.3 ± 0.1
Females	Healthy	956	50.7 ± 2.2	43.8 ± 2.0	2.9 ± 0.4[a]	2.4 ± 0.4	0.2 ± 0.1
	F. hepatica	914	46.5 ± 4.1	42.1 ± 3.8	8.4 ±2.3[b]	2.3 ± 0.4	0.8 ± 0.4

Source: Authors' own elaboration.
Note: [a, b] Different subcripts indicate significant differences ($P < 0.05$).

Table 2: White blood cells counts of bovines grouped according to the number of flukes in the liver

	No. of flukes	n	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)	Basophils (%)
Males	Healthy*	992	54.9 ± 1.6[a]	38.7 ± 1.5[b]	2.0 ± 0.3[a]	3.8 ± 0.4[a]	0.6 ± 0.1
	1 – 10	249	50.4 ± 5.3[ab]	42.2 ± 4.0[b]	4.8 ± 0.26[b]	2.6 ± 0.7[ab]	0.0 ± 0.0
	11 – 25	301	49.8 ± 2.8[ab]	43.0 ± 3.7[ab]	5.5 ± 0.17[b]	1.3 ± 0.6[c]	0.5 ± 0.3
	26 – 50	154	54.5 ± 0.5[ab]	41.5 ± 1.5[b]	6.5 ± 0.5[b]	1.5 ± 1.5[b]	0.0 ± 0.0
	>51	292	31.0 ± 1.0[b]	64.0 ± 1.0[a]	6.5 ± 0.4[b]	1.5 ± 0.5[b]	1.0 ± 0.0
Females	Healthy	956	50.7 ± 2.2	43.8 ± 2.0	2.9 ± 0.4[b]	2.4 ± 0.4[a]	0.2 ± 0.1
	1 – 10	436	50.5 ± 6.9	44.4 ± 6.0	3.0 ± 0.7[b]	1.9 ± 0.6[b]	0.3 ± 0.2
	>11	478	45.0 ± 2.0	45.5 ± 5.5	8.0 ± 0.8[c]	1.5 ± 0.5[c]	0.0 ± 0.0

Source: Authors' own elaboration.
Notes: a * Healthy animals had no flukes and normal metabolic profiles.
b [a,b,c] Different subcripts indicate significant differences ($P < 0.05$).

Metabolic profile

In relation to metabolic profile (Table 3), we observed significant differences ($P < 0.05$) in the values of BUN (greater for healthy animals) and phosphorus (greater among affected animals) between healthy and infected males. Females differed only in BUN (greater for healthy animals).

Based on the metabolic profile of animals with fascioliasis grouped according to the number of flukes (Table 4), we observed in males that the values of BUN decreased as the number of flukes in the liver parenchyma increased. A similar trend was observed in females, although it was less pronounced, perhaps in part because of less extreme groups for numbers of flukes in females. The concentrations of cholesterol, TP, ALT and AST generally decreased in males as the number of flukes increased, while in females a trend was observed for only AST (Table 4).

Table 3: Values of elements contributing to the metabolic profile of clinically healthy bovines and bovines with *F. hepatica*

		n	Calcium mg/dL	Phosphorus mg/dL	BUN mg/dL	Bilirubin mg/dL	Cholesterol mg/dL	Total proteins mg/L	γGT (U/l)	ALT (U/l)	AST (U/l)	LHD (U/l)	Alkaline phosphatase (U/l)
Males	Healthy	992	8.4 ± 0.5	5.2 ± 0.01[a]	26.4 ± 1.5[a]	0.2 ± 0.01	182.9 ± 19.4[a]	49.8 ± 1.9	28.5 ± 4.7	258.1 ± 66.6	131.8 ± 39.5[a]	1838 ± 88.4[a]	375.8 ± 39.3
	F. hepatica	996	8.8 ± 0.4	5.5 ± 0.1[b]	13.9 ± 1.9b	0.3 ± 0.02	163.8 ± 22.6[b]	47.1 ± 1.7	29.3 ± 5.1	259.7 ± 51.6	51.9 ± 20.4[b]	1338 ± 227.4[b]	431.3 ± 41.5
Females	Healthy	956	8.2 ± 0.5	5.3 ± 0.1	22.8 ± 2.6[a]	0.4 ± 0.08	161.3 ± 28.5	52.5 ± 1.8	26.4 ± 6.3	258.3 ± 44.3	68.2 ± 28.9	1980 ± 155.1[ab]	250.9 ± 48.1
	F. hepatica	914	8.1 ± 0.3	5.3 ± 0.1	14.9 ± 1.6[b]	0.3 ± 0.02	162.7 ± 16.4	47.4 ± 2.9	22.9 ± 2.3[b]	245.5 ± 44.9	67.7 ± 17.4	1500 ± 192.9[b]	404.8 ± 59.9

Source: Authors' own elaboration.
Notes: a * Healthy animals had no flukes and normal metabolic profiles.
b [a,b] Different subcripts indicate significant differences ($P < 0.05$).

Table 4: Values of elements of the metabolic profile of bovines grouped according to the number of flukes in the liver

	No. of flukes	n	Calcium mg/dL	Phos-phorus mg/dL	Urea/ BUN mg/dL	Bilirubin mg/dL	Cho-lesterol mg/dL	Total proteins mg/L	γGT (U/l)	ALT (U/l)	AST (U/l)	LHD (U/l)	Alkaline phospha-tase (U/l)
Males	Healthy	992	8.4 ± 0.5	5.2 ± 0.01	26.4 ± 1.5^{a}	0.25 ± 0.01	182.9 ± 19.4^{a}	49.8 ± 1.9^{a}	28.5 ± 4.7^{a}	258.1 ± 66.6^{a}	131.8 ± 39.5^{a}	1 838 ± 88.4^{a}	375.8 ± 39.3^{a}
	1 – 10	249	8.8 ± 0.6	5.4 ± 0.2	17.4 ± 3.2^{ab}	0.29 ± 0.02	193.6 ± 38.5^{a}	42.8 ± 1.2^{b}	17.4 ± 2.9^{b}	239.1 ± 41.2^{a}	77.5 ± 56.1^{b}	1 048.3 ± 498.8^{c}	490.9 ± 69.5^{b}
	11 – 25	301	9.2 ± 0.2	5.4 ± 0.1	18.7 ± 1.2^{ab}	0.28 ± 0.01	199.2 ± 21.6^{b}	50.7 ± 0.5^{a}	23.2 ± 2.9^{ab}	151.7 ± 11.7^{b}	59.4 ± 13.1^{b}	1 740.4 ± 129.5^{a}	490.6 ± 20.2^{b}
	26 – 50	154	10.9 ± 0.4	5.8 ± 0.02	13.5 ± 1.1^{ab}	0.28 ± 0.01	143.2 ± 12.4^{b}	45.4 ± 3.6^{b}	29.9 ± 1.6^{a}	155.1 ± 7.8^{b}	60.2 ± 3.5^{c}	2 064.2 ± 324.1^{b}	371.2 ± 24.5^{a}
	>51	292	8.9 ± 0.5	5.4 ± 0.02	9.2 ± 1.4^{b}	0.24 ± 0.01	97.8 ± 8.9^{c}	39.6 ± 3.8^{c}	29.6 ± 0.9^{a}	60.2 ± 5.3^{c}	11.3 ± 2.1^{d}	1 440.9 ± 232.2^{c}	151.8 ± 13.4^{c}
Females	Healthy	956	8.2 ± 0.5	5.3 ± 0.1	22.8 ± 8.2^{a}	0.37 ± 0.08	161 ± 28.5^{ab}	52.5 ± 1.8^{a}	26.4 ± 6.3^{ab}	258.3 ± 44.3^{a}	68.2 ± 28.9^{b}	1 980.4 ± 155.1^{a}	250.9 ± 48.1^{b}
	1 – 10	436	7.6 ± 0.4	5.2 ± 0.1	16.9 ± 2.4^{b}	0.27 ± 0.03	171.7 ± 31.3^{ab}	49.2 ± 3.3^{a}	22.4 ± 2.6^{ab}	230.6 ± 77.1^{ab}	73.9 ± 36.4^{b}	1 455.5 ± 152.6^{ab}	505.4 ± 88.8^{a}
	11 – 25	478	8.2 ± 0.6	5.4 ± 0.2	18.2 ± 3.2^{ab}	0.31 ± 0.02	163.5 ± 7.9^{ab}	58.9 ± 4.9^{a}	29.7 ± 8.4^{a}	300.0 ± 114.8^{a}	47.8 ± 16.3^{c}	1 413.6 ± 458.4^{ab}	357.4 ± 26.1^{ab}

Source: Authors' own elaboration.

Notes: a * Healthy animals had no flukes and normal metabolic profiles.

b ^{a,b,c} Different subcripts indicate significant differences ($P < 0.05$).

4. Conclusions

The study demonstrates the significant damage caused by this parasitic disease (fascioliasis or dystomatosis) in ruminants of economic importance. It is estimated that more than 182 million dollars are lost in Mexico by this disease annually, due to treatments, low productivity and reproductive disorder in creole cattle, of which, despite the inclement weather and poor feeding procedures, these animals constitute 62 percent of the meat and milk production nationwide. Fascioliasis constitutes an important parasitic disease in Mexico that cause damage to liver and increase the incidence of other diseases such as cholangiohepatitis, hepatitis, liver tumours, cholangiectasis, fibrosis, cirrhosis, liver cancer among others, and finally affects metabolic profiles of the most important blood metabolites liver and pancreatic enzymes. This parasitosis ultimately affects the animal welfare of creole cattle.

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Characterization of the Foot-and-Mouth Disease virus in Burkina Faso: Outbreaks of 2018

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Abstract

Foot-and-Mouth Disease is an enzootic disease in Burkina Faso. Many suspected outbreaks are notified each year, but very few are confirmed by laboratory analysis and characterization studies. This is a characterization study of the virus conducted in four localities in four regions of the country during the outbreak of the disease in 2018. A total of 18 samples of canker sore vesicular fluid and canker sore swabs were collected and analysed. The isolated virus belonged to serotypes O and to the topotype EA-3. Routine characterization of the virus during future outbreaks would allow for more effective and efficient control of the disease in Burkina Faso.

Keywords

Foot-and-Mouth Disease, virus, characterization, outbreak, cattle, Burkina Faso

1. Introduction

Foot-and-Mouth Disease is a viral disease that affects cattle, buffaloes, pigs, sheep, goats and a variety of wildlife. The disease can seriously affect animal production and significantly disrupt regional and international trade in animals and their products.

Foot-and-Mouth Disease virus belongs to the Picornaviridae family and to the Aphthovirus genus. The viral particle has an icosahedral symmetry and is formed by the assembly of 60 copies of the four capsid proteins (VP1, VP2, VP3 and VP4) around an RNA molecule of positive polarity (Acharya *et al.*, 1989).

The capsid surface is marked by the presence of a mobile protuberance formed by the G-H loop of the VP1 protein. This G-H loop is the target of many neutralizing antibodies and is also involved in the interaction of the virus with its cellular receptor (Neff *et al.*, 1998). This region permits the classification of the virus into one of seven immunologically distinct serotypes circulating around the world (O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1). All these serotypes are found in Africa except Asia 1. In West Africa, we typically find the serotypes O, A, SAT 1 and SAT 2. Within each serotype, multiple subtypes or variants have been identified.

Foot-and-Mouth Disease is enzootic in Burkina Faso. From January 2014 to June 2018, 384 outbreaks were reported to the World Organization for Animal Health (WOAH). In 2018, the severity of the disease caused heavy losses on farms. Vaccination is not yet implemented in the country and the control measures of Foot-and-Mouth Disease applied consist primarily of the implementation

of biosecurity in dairy farms. The objective of this study was to determine the serotypes of the Foot-and-Mouth Disease virus on the basis of outbreaks in four regions of Burkina Faso in 2018.

2. Material and method

Study area

The study involved four localities in four regions (Ziniaré in Plateau Central region, Sabou in Centre Ouest region, Safané in Boucle du Mouhoun region and Saaba in Centre region) out of the 13 regions that make up the territory of Burkina Faso. These areas were identified on the basis of bovine mortality alerts and presence of animals showing clinical signs (i.e. unbroken vesicles, tongue ulceration, etc.).

Sampling

A total of 18 samples were taken from farms with clinical suspicion of this disease. Only notified outbreaks were included. The samples were of vesical fluid, epithelial fragments and buccal swab made from sick animals. Table 1 summarizes the types of samples and the locations where they were collected.

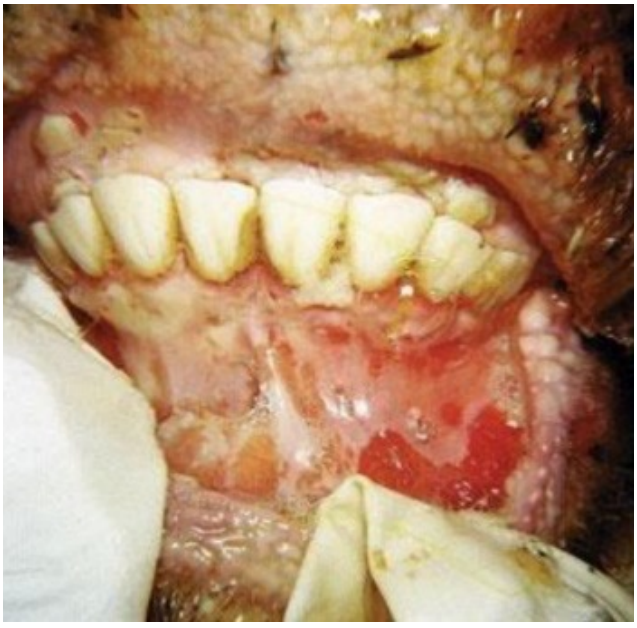
Table 1: Sample collection by locality

Registry code	Sample identification number	Sample type	Locality	Number of samples
18/248	18/248_BKF01	Vesicular fluid and epithelial fragments	Centre-Ouest/ Boulkièmdé/Sabou	1
	18/248_BKF02 to 18/248_BKF04	Buccal swab		3
18/249	18/249_BKF05 to 18/249_BKF13	Buccal swab	Centre/Kadiogo/Saaba	9
18/250	18/250_BKF14 to 18/250_BKF15	Buccal swab	Plateau Central/ Oubritenga/Ziniaré	2
18/269	18/269_BKF16 to 18/269_BKF18	Buccal swab	Boucle du Mouhoun/ Lanfiéra/Safané	3
Total of samples				18

Source: Authors' own elaboration.

Collection and transportation of samples

The vesicular fluid was taken from animals with unbroken vesicles. Figure 1 shows an example of lesions characteristic of Foot-and-Mouth Disease. The procedure consisted of mechanically restraining the affected animal, and then either using a syringe to withdraw the contents of the vesicular fluid or inducing a tear in the surface of the vesicle and removing the fluid contents by swabbing. In the event where a vesicle had already ruptured already, the vesicular skin fragments were removed with sterile medical pliers.



Source: Author's own elaboration.

Figure 1: Lesions of Foot-and-Mouth Disease in the mouth of a cow

Samples were triple wrapped for packaging and placed in coolers for shipping. They were transported by public transport companies to the Laboratoire National d'Elevage in Ouagadougou with an average transport time of 2 hours. In the event of unavailability of immediate transport, the samples were stored overnight on site at -20 °C and then shipped to the Laboratoire National d'Elevage no later than 24 hours after collection. Samples were stored there at -80 °C for no more than 30 days before shipment to the Pirbright Institute in England, the WOAH reference laboratory for Foot-and-Mouth Disease. Sequencing of the samples was performed by staff of the Pirbright Institute in the United Kingdom to allow for molecular characterization.

Isolation and genotypic characterization

Virus isolation and genotypic characterization were performed at the Pirbright Institute. Primers O-1C244F/EUR-2B52R; O-1C272F/EUR-2B52R; and FMD-3161F/FMD-4303R were used. Twelve (12) samples were positive based on successful foot-and-mouth virus genome amplification. Phylogenic analysis was performed by using the parameters shown in Table 2.

Table 2: Phylogenic analysis parameters

Parameter	Values
VP1 subsequence extractor:	vp1_O
Query sequence set:	WRLFMD/2018/00031-Burkina Faso-O (7 sequences)
Sequence database set:	allseqs_O (3 926 sequences)
Prototype sequence set:	!prototypes_O (49 sequences)
Number of related sequences reported:	10
Minimal VP1 subsequence match length	600
Sequence alignment method:	muscle (default parameters)
Sequence alignment length:	636
Phylogeny reconstruction method:	fdnadist, fneighbor
Number of bootstrap samples:	1 000
Random seed for bootstrapping:	1
Displaying bootstrap values above:	70.0%
Number of sequences in tree capped at:	100
Number of prototype sequences in tree:	13
VIBASys version:	reflabs-1.1.2

Source: Authors' own elaboration.

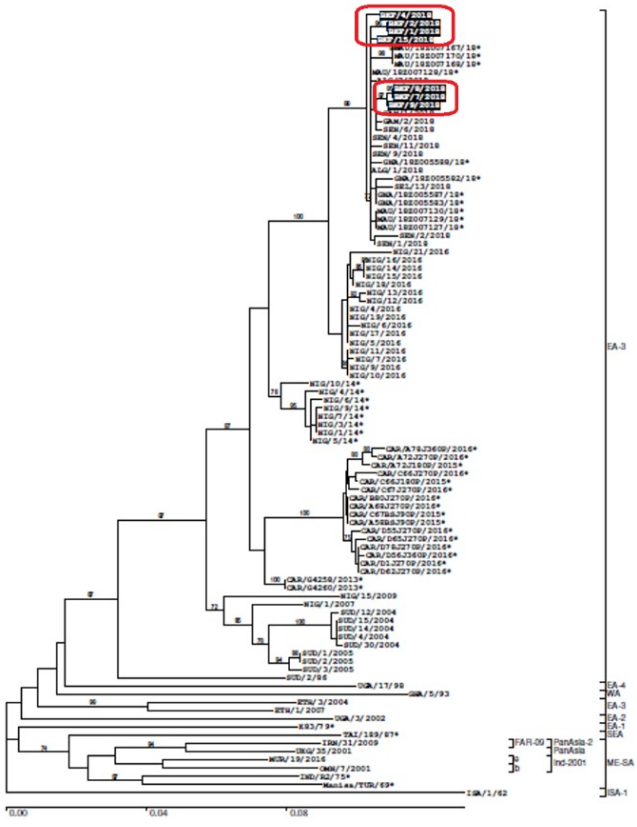
3. Results

The characterization study revealed that the isolated viruses belonged to serotypes O and to the topotype EA-3. The phylogenetic tree is shown in Figure 2. Genotyping reports (EuFMD, 2020) have revealed the same serotype and topotype for Senegal, Mauritania, Gambia, Algeria in 2018 and Nigeria in 2014 and 2016.

4. Discussion

The analysis indicated that the circulating virus was of serotype O, topotype EA-3. This strain occurs with clinical manifestations ranging from mild to severe (Soltan *et al.*, 2017). The topotypes EA-3 and EA-2 are responsible for most of the outbreaks observed on the Africa (Lloyd-Jones *et al.*, 2017). The serotype O circulates in many countries in West Africa (Teye *et al.*, 2019) and is panzootic in the region (FAO, 2017; McLachlan *et al.*, 2019). From 1990 to 2013, serotypes O, A, SAT1 et SAT 2 were found in Africa (Paton, Sumption and Charleston, 2009). The dominant farming systems, which include frequent cattle movements and contact with wildlife, play a critical role in the transmission of the virus (Rweyemamu *et al.*, 2008). In Burkina Faso the main farming method is transhumance with a very low application of vaccination. Mixed livestock farming with various species is common as well, and also favours the maintenance and propagation of the virus.

Investigation of all outbreaks each year is necessary to determine all serotypes circulating in the country. Knowing the circulating serotypes of the Foot-and-Mouth Disease



Source: Author's own elaboration.

Figure 2: Phylogenetic analysis of Foot-and-Mouth Disease virus from Burkina Faso: Neighbour-joining tree, of VP 1 gene region

virus is necessary because it allows to define the valency that the vaccine against the disease should contain. As cross-immunity does not exist in the case of Foot-and-Mouth Disease, this allows an effective and efficient humoral response of the animals against all serotypes of the virus circulating in the country.

Moreover, the greater the valency of the vaccine, the greater its cost. Using only the vaccines specific only for the serotypes found in the country would reduce the cost of the vaccine and make it accessible to small producers, who are most often the most exposed in a transhumant livestock context. These producers are also potential reservoirs of the virus because of the contact of their herds not only with the herds of neighbouring countries but also with wild animals.

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Identification of H5N1 avian influenza virus from poultry farm and live bird markets in Banten, West Java and Central Java Province, Indonesia, 2018–2019

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Abstract

Live bird market (LBM) and poultry farms are considered to play a role in the transmission of avian influenza virus (AIV) between poultry and humans. For example, AIVs have been discovered in several LBMs and poultry farms in Indonesia. In this study, AIV surveillance was conducted in three provinces in Indonesia (Banten, West Java, and Central Java) from 2018 to 2019. The AIV H5N1 subtype (AIV-H5N1) was found in one poultry farm and three LBMs. About 19 percent (N=50) of all samples (N=268) were positive for influenza type A and subsequently, about 34 percent of these influenza type A positive samples were positive for H5 subtyping. Samples confirmed positive for A/H5 were further subtyped for the N1 gene and 58.8 percent were positive. Accordingly, 3.73 percent isolates tested positive for the H5N1 subtyping test. The study concluded that AIV-H5N1 is still circulating in poultry farms and LBMs in Banten, West Java and Central Java Province. Related to its potential infecting humans, pandemic preparedness activities, such as monitoring, surveillance and biosecurity measures in farms and LBMs should be implemented comprehensively.

Keywords

live bird market, avian influenza, Indonesia, poultry farm

1. Introduction

In Indonesia, the first outbreak of avian influenza virus (AIV) was found in commercial and backyard domestic poultry flocks in 2003 (Dharmayanti *et al.,* 2004). The causative agent of the outbreak was confirmed to be highly pathogenic avian influenza (HPAI) A(H5N1) virus through field examination, clinical symptoms, pathological study, immunohistochemistry, and reverse transcription-polymerase chain reaction (RT-PCR) screening (Dharmayanti *et al.*, 2004; Wiyono *et al.*, 2004). Influenza A viruses are RNA-enveloped viruses with an eight-segmented, single-stranded, negative-sense genome belonging to the family *Orthomyxoviridae.* The eight-segmented genome encodes for ten viral proteins: HA, NA, M1, M2, NP, NS1, NEP, PB1, PB2, and PA (Nuñez and Ross, 2019). The mutations can occur due to errors during the replication of viral RNA polymerase, evolutionary pressure, the novel environment of the host, immune pressure or antiviral drug pressure (Shao *et al.,* 2017). The segmented genome of influenza viruses also allows genetic reassortment when two influenza viruses infect the same cell (co-infection) (Peiris, de Jong and Guan, 2007).

Avian influenza type A viruses are divided into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) based on their pathogenicity in chickens (Mosad *et al.,* 2020). The spread of AIV usually occurs very rapidly and has been reported in the majority of Indonesia's provinces with the exception of Papua, Maluku and North Maluku (Director-General of Livestock and Animal Health, 2020). In 2012, an HPAI H5 outbreak was found to infect ducks with a high mortality rate (Dharmayanti *et al.,* 2013). Live bird markets (LBMs) play a role in the transmission of AIV between poultry and humans, as AIVs have been detected in several LBMs in Indonesia (Perdue and Swayne, 2005; Hartawan and Dharmayanti, 2014; Dharmayanti *et al.*, 2020). The first case of bird flu in humans occurred in Tangerang, Banten in 2005 (Kandun *et al.*, 2006). In 2012, Indonesia reported a fatal case of AIV infection in human in an 8-year-old girl (Naysmith, 2014). The infection likely occurred when she visited a LBM and had close contact with live poultry (Naysmith, 2014). From 2003 to 2020, there were 862

cases of human infection with AIV-H5N1 reported from 17 countries, with Indonesia having the highest mortality rate in the world (168 deaths from 200 cases) (WHO, 2021).

Due to the rapid evolution of AIV, several genetic reassortment events have been reported in Indonesia. Recently, Dharmayanti *et al.* (2020) reported on the genetic diversity of the H5N1 viruses in live bird markets in Indonesia, including the presence of reassortant H5N1 viruses between clades 2.1.3 and 2.3.2. Reassortant viruses have also been detected between the LPAI and HPAI virus subtypes in Indonesia (Dharmayanti, Indriani and Nurjanah, 2020). Besides LBM, AIV-H5N1 has been proven to contaminate all types of avian production, environments and species, including commercial poultry farms, wild birds, backyard domestic animals, live poultry, game birds and mixed poultry (Chatziprodromidou *et al.,* 2018). In poultry farms, contamination can occur through the movement of people, vectors, contaminated environmental components (air, water and dust) and other objects (e.g. cages, staff equipment and vehicles) (Singh *et al.,* 2018). In some cases of AIV infection in humans, close contact with infected birds in poultry farms has also been reported (Ali *et al.,* 2019). Vaccination pressure and co-circulation among AIV subtypes/clades on the poultry farm may contribute to the evolution of AIV (Abdel-Moneim, Afifi and El-Kady, 2011; Parvin *et al.,* 2018).

Due to the discovery of antigenic drift and antigenic shift in AIVs in Indonesia, monitoring and surveillance activities need to be carried out to understand the circulation and distribution of AIV in the field (Dharmayanti *et al.,* 2011a; Dharmayanti *et al.,* 2011b; Dharmayanti *et al.,* 2018). This study aimed to identify the circulation of AIV-H5N1 in LBMs and poultry farms in Banten, West Java and Central Java Province in 2018–2019, which will be used as a base reference for further research in control measures of AIV cases in Indonesia.

2. Methods and materials

Sampling collection

Sample collection was performed on poultry farms and LBMs in which people sell and transport live poultry as well as on poultry products from several large cities or regencies in the provinces of Banten (Serang City), West Java (Sukabumi Regency and Cianjur Regency), and Central Java (Brebes Regency and Tegal Regency) in 2018–2019 (Table 1). Sampling sites were subjectively characterized by taking note of species, confinement facilities, transport methods, slaughtering facilities, modes of interaction with shoppers, and other features of potential relevance for viral transmission. The environmental samples were taken from the floor, knives, chopping boards, weighing scales, de-feathering machines, tables for poultry display, waste bins, and used clothing prior to cleaning. Dead birds that had shown clinical symptoms were necropsied and their organs were taken as samples. Swabs were taken from the cloacae of the live birds and pooled within the same species (maximum three samples). The cloacal, organ and environmental samples were placed in transport medium (Dulbecco's modified Eagle's medium; GIBCO, Thermo Fisher Scientific, USA) and maintained in a portable refrigerator freezer (−20 °C) during transportation to the Indonesian Research Center for Veterinary Science, Bogor, Indonesia.

Virus identification

Cloacal swabs and environmental samples in the viral transport medium were processed for viral RNA isolation using the QIAmp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturers' instructions. Organ samples ($N = 3$) were processed using the RNeasy Mini Kit (Qiagen, Germany) according to the kit instructions. RT-PCRs were carried out to identify the AIV A group with the matrix gene target with the primer set sequences M52C (5-CTT CTA ACC GAG GTC GAA ACG-3) and M253R (5-AGG GCA TTT TGG ACA AAG/T CGT CTA-3) with a 244 bp amplicon (Fouchier *et al.,* 2000). The amplicon was separated by agarose gel electrophoresis using a 2.0 percent gel run at 100 V for 30 minutes and then visualized with a UV-Vis transilluminator. The AIV type A-positive samples were confirmed using RT PCR for H5 subtyping (Dharmayanti, Hartawan and Hewajuli, 2016). The RT-PCR reaction was performed with a 9800 Fast Thermal Cycler Applied Biosystems machine (Qiagen, Hilden, Germany) using Superscript III One-Step RT-PCR system by Life Technologies (Waltham, MA, USA) with a reaction mixture of 10 µL RNA as the template, 2 µL for each primer, 1 µL Taq Polymerase enzyme, 25 µL PCR Master Mix (2X), and NFW (nuclease-free water) up to 50 µL. RT-PCR was performed using the thermal cycle conditions as follows: reverse transcription at 42 °C for 45 min; initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 95 °C for 30 s; annealing at 50 °C for 40 s; extension at 72 °C for 40 s; and final extension at 72 °C for 10 min. The H5-positive samples were cultured in specific pathogenic free 9–11 day old embryonated eggs (WOAH, 2021). The allantoic fluids of H5 positive sample was harvested and kept at 20 °C for further analysis. For all samples that were confirmed positive for A/H5, a subtyping for the N1 gene was performed (Wright *et al.,* 1995).

DNA sequencing and Bioinformatics analysis

The amplification of NA and partial HA gene of AIV-H5N1 was conducted by following previous methodology (Dharmayanti *et al.,* 2018; Dharmayanti *et al.*, 2020; Dharmayanti, Indriani and Nurjanah, 2020). The PCR products were separated in 1 percent agarose by electrophoresis and the amplicon was excised and purified

using a QIAquick gel purification kit (QIAGEN, Hilden, Germany). The nucleotide sequencing data obtained in this study were analysed together with the genetic data available from the National Center for Biotechnology Information (NCBI) of the United States of America. The sequencing results were verified and edited using BioEdit version 7.1.3.0 (www.mbio.ncsu.edu/BioEdit). Phylogenetic trees were generated using Maximum Likelihood with 1.000 bootstrap support using the Tamura-Nei algorithm in MEGA X.

3. Result and discussion

Table 1 shows the distribution of species on the various farms and LBMs in the Districts where samples were collected. The sellers at LBM offer various types of live poultry such as chickens, ducks, Muscovy ducks, geese, and pigeons and their products as well as slaughtering services. The LBMs are usually close to the slaughterhouse that provides services within the market and accepts live bird processing from neighbouring areas. Several sellers regularly transport the live poultry with modified motorcycles with baskets made of wood or bamboo, then serve buyers at the market by selling various species of birds in one basket on the motorbike (see Figure 1). In many stalls, different types of birds of various ages are mixed in one place. Moreover, visitors/buyers can directly choose by physically handling and inspecting the poultry they want to buy without wearing a mask or similar protection. This practice is considered to create a high potential for the spreading the virus from birds to humans (Dharmayanti *et al.*, 2011a; Naysmith, 2014). Close contact between birds and humans in LBM can lead to the transmission of various zoonotic diseases. Some of the zoonotic disease transmissions associated with live animal markets are avian influenza and Severe Acute Respiratory Syndrome (SARS) (Magouras *et al.*, 2020).

Among the poultry farms that we sampled in this study, some had experienced high mortality rates with clinical symptoms that include weakness, lethargy, loss of appetite and torticollis. Wild and feral ducks in the surrounding area also experienced frequent illness and deaths. In this case, although disinfection is carried out regularly, the application of biosecurity has not been properly implemented, such as the separation of sick and healthy chickens, traffic control, and separation of poultry by age. Accordingly, several points that need to be considered in the management of AIV in poultry farms include the type of poultry production, vaccination status, level of biosecurity, the presence of other pathogens, the number of visitors to the farms, the turnover of poultry production, the extent of inward (inputs) and outward (outputs) movements, or the position of the farm in the poultry value-chain network (Artois *et al.*, 2018).

Source: Author's own elaboration.

Figure 1: The interactions that occur between various birds and humans in live bird markets in Indonesia: (A) poultry slaughterhouse in the market having close contact with live poultry in stalls as well as with humans; (B) a modified motorcycle with a basket as a tool for transporting various poultry individuals and species; (C), poultry stall where various poultry species are mixed including chickens, ducks and Muscovy ducks; (D) close contact that occurs between humans and various species of poultry at market stalls

Table 1: Poultry species present on the farms and live bird markets where samples were collected

No.	City/Regency	Location	Species	Years of sample collection
1.	Tegal Regency	Farm1	Layers	2019
		Farm 2	Layers	
		Farm 3	Ducks	
		Farm 4	Ducks	
		LBM	Domestic chickens, geese, Muscovy duck, broilers, environment	
2.	Brebes Regency	Farm 1	Layers	2019
		Farm 2	Layers	
		Farm 3	Ducks	
		Farm 4	Ducks	
		LBM	Domestic chickens, layers, duck, Muscovy duck, environment	
3.	Sukabumi Regency	Farm 1	Domestic chickens, layers	2018
		Farm 2	Layers	
		Farm 3	Ducks	
		Farm 4	Ducks, Muscovy ducks	
		LBM	Domestic dhickens, ducks, Muscovy ducks	
4.	Cianjur Regency	Farm 1	Domestic chickens, ducks, Muscovy ducks	2018
		Farm 2	Domestic chickens	
		Farm 3	Domestic chickens	
		Farm 4	Layers	
		LBM	Domestic chickens, ducks, Muscovy ducks, layers	
5.	Serang City	Farm 1	Domestic chicken	2019

Source: Authors' own elaboration.

As depicted in Table 2, 18.65 percent of samples were positive for AIV A and, subsequently, about 34 percent of these positive AIV A samples were positive for H5 subtyping. Among the 52 samples from Brebes Regency tested for RT-PCR influenza type A viruses, 15 (28.8 percent) were positive, including 8 (15.4 percent) that were AIV A/H5 positive. In Tegal Regency, 38 samples were tested for AIV A and 2 (5.2 percent) were positive, including 1 (2.63 percent) that was AIV A/H5 positive. Out of 89 samples from Sukabumi Regency, 23 were positive for AIV A viruses (25.8 percent), including 1 (1.1 percent) that was H5 positive. In Cianjur Regency, out of 82 samples, three were positive for AIV A viruses (3.6 percent), with no samples testing positive for H5. Finally, out of 10 samples from Serang, Banten, 7 were positive for AIV A/H5 (70 percent).

When considering the environmental samples, which were taken in only the Brebes and Tegal Districts, 15.8 percent ($N = 3$) of the samples were positive for AIV A and all samples positive for AIV type A were also found to be positive on H5 subtyping. For all samples that were confirmed positive for A/H5, a subtyping for the N1 gene was performed. The results showed that, of the 17 samples that were positive for A/H5, 10 of them were positive for the N1 gene (58.8 percent).

Furthermore, seven isolates that were positive for H5 subtyping tested negative for N1. The isolate condition, primer binding site mutation, and various AIV subtypes can be considered as factors that can affect the results of the test. Among the ten samples that were positive for AIV-H5N1, two isolates (A/chicken/Indonesia/BBLitvet-SRA07/2018 and A/chicken/Indonesia/BBLitvet-SRA10/2018) were used in a phylogenetic analysis on their NA and partial HA genes together with the sequences available in NCBI (Figure 2). The NA and partial HA genes from each of these viruses were grouped with those from related AIV-H5N1 Indonesian viruses obtained from similar geographic locations and still in the same group as the Indonesian isolate.

As shown in Table 1, various types of poultry are found on LBMs. The LBM is considered as an ideal environment for genetic mixing and transmission of AIV because its reservoirs, waterfowl, are sold together with other poultry. In Asia, several subtypes of AIV type A viruses are simultaneously found on LBM. This condition allows poultry to be infected by viruses from several subtypes or clades (co-infection) and the subsequent production of virus progeny with antigenic shifts (reassortment) (Dharmayanti *et al.*, 2018; Dharmayanti *et al.*, 2020; Dharmayanti, Indriani and Nurjanah, 2020). In poultry farms, mutations can occur by immunological pressures

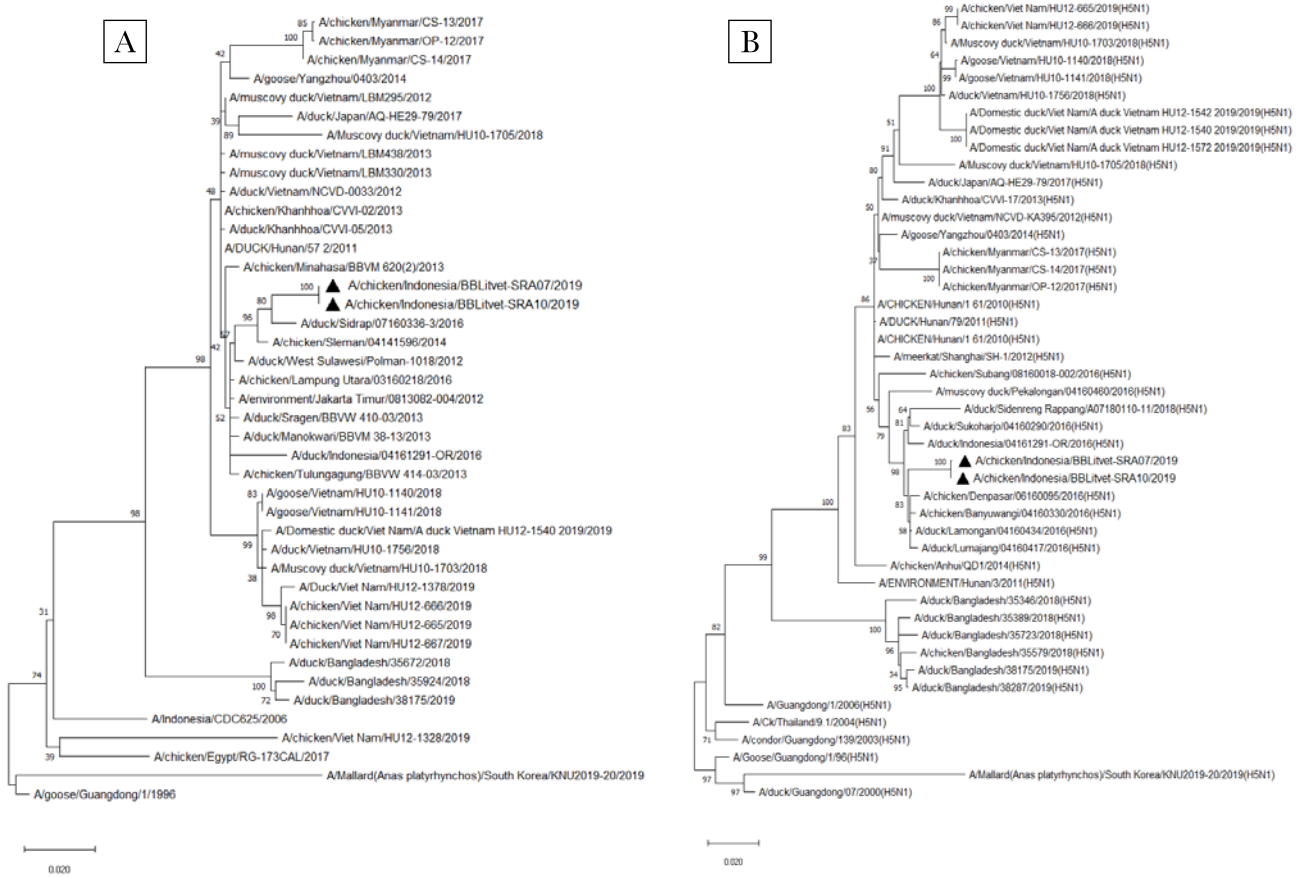
from the vaccination or antiviral drugs (Dharmayanti and Darminto, 2009; Abdel-Moneim, Afifi and El-Kady, 2011; Dharmayanti *et al.*, 2011b).

The findings of this study provide sufficient evidence that AIV-H5N1 contamination is common in the LBMs and poultry farm environments in several provinces of Indonesia. Viral contamination can derive from droplets from slaughtering, removing feathers, and cleaning the carcasses of infected poultry, especially internal organs that might have high viral loads. Poor management of LBMs, such as the absence of regular rest days to clean and disinfect stall/slaughter premises, causes severe hygiene problems and potential virus spreading in LBMs (Wang *et al.*, 2017). On the other hand, poultry farm contamination can also occur through the movement of people, vectors, the contaminated environment (air, water and dust) and other objects (e.g. cage equipment and vehicles) (Singh *et al.*, 2018).

Table 2: RT-PCR test result for H5NI subtyping in Indonesia

No.	Location	Sample swab	Number of samples tested for influenza type A	RT-PCR Positive result		
				Influenza type A	H5	N1
1	Brebes Regency	Cloacal (Pool)	36	10	4	1
		Organ (Individual)	3	3	2	0
		Environmental (Individual)	13	2	2	0
2	Tegal District	Cloacal (Pool)	32	1	0	n.d
		Environmental (Individual)	6	1	1	1
3	Sukabumi District	Cloacal (Pool)	89	23	1	1
4	Cianjur District	Cloacal (Pool)	82	3	0	n.d
5	Serang, Banten	Cloacal (Pool)	6	6	6	6
		Organ (Pool)	1	1	1	1
		Total sample	**268**	**50**	**17**	**10**

Source: Authors' own elaboration.
Note: n.d: no data.



Source: Author's own elaboration.

Figure 2: Phylogenetic tree of the partial HA gene (A) and (B) NA gene of H5N1 Subtype Avian Influenza; the isolates used in this study are indicated by black triangle shapes

The AIV-H5N1 has also been reported to have the ability to replicate in the digestive tract (Shu *et al.*, 2010), thus consumption of contaminated food is also a potential source of pathogen transmission. In this matter, the location of the food stalls of LBMs, which is usually nearby poultry stalls and the slaughterhouse, is an important point that needs to be considered to prevent viral transmission to humans. Moreover, a study by Horm *et al.* (2012) reported that AIV-H5N1 can survive outside the host for a few days up to several months depending on the environmental conditions and viral concentrations. Its viral RNA has also been detected in environmental specimens such as mud, pond water, aquatic plants and soil/dust swabs (Horm *et al.*, 2012).

The AIV-H5N1 in Indonesia has experienced antigenic drift since 2006, with an estimated 1 percent of its amino acids changing every year (Dharmayanti and Darminto, 2009; Dharmayanti *et al.*, 2011b). Various reassortment events have also been reported in Indonesia, with reassortments occurring among AIV clades or subtypes (co-infection) (Dharmayanti *et al.*, 2018; Dharmayanti *et al.*, 2020; Dharmayanti, Indriani and Nurjanah, 2020). The mutation of the AIV may complicate the detection of AIV-H5N1 in the field. Because of the ability AIV-H5N1 evolution, it is crucial to evaluate the appropriate methods used to detect and diagnose AIV-H5N1 in the field, such as regularly designing new primers based on current circulating virus. For this reason, further investigation is needed to determine mutations that occur in AIV. Furthermore, inability to identify the AIV-H5N1 can also be caused by the differences in virus subtypes. Therefore, further subtyping tests may need to be performed.

4. Conclusions

The study concludes that AIV-H5N1 is still circulating within poultry farms and LBM in several Indonesian provinces, namely Banten, West Java and Central Java. Due to rapid evolution of AIVs, regularly designing novel primer based on the current circulating AIV must be considered for better diagnostics. In addition, due to the discovery of antigenic drift and antigenic shift in AIVs in Indonesia from previous study (Dharmayanti *et al.*, 2011b; Indi Dharmayanti, 2012; Dharmayanti *et al.*, 2018; Dharmayanti *et al.*, 2020; Dharmayanti, Indriani and Nurjanah, 2020), monitoring and surveillance activities need to be carried out periodically and simultaneously to better understand the circulation and distribution of the AIVs in the field.

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Enhancing livestock's contribution to one health and the SDGs

Health hazards in animal feed: Highlights from a recent FAO report

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Abstract

Animal feed is a potential source of hazards affecting animal health and, after transformation to food, human health. This important role of animal feed has been acknowledged by international organizations such as the Food and Agriculture Organization of the United Nations (FAO), the International Organization for Animal Health (WOAH), Codex Alimentarius, and the International Feed Regulators. These hazards may enter and persist in the food production chain at various stages, such as raw material sourcing, feed production, consumption of pastures, fresh and processed feeds by livestock, metabolism by the animal, transfer to edible tissues, and processing of the animal food product. Two FAO/WHO joint expert meetings were convened on this topic in 2007 and 2015, the latter providing an update and review to the first one. The recommendations that emanated from the 2015 meeting straddled the different fields of guidance for hazard prevention and control, capacity development, international standards, awareness within the sector, risk assessment, monitoring, enforcement, and research needs. The items considered in detail in the meeting report and background document included general hazards (chemical, biological, physical), hazards of increasing relevance (e.g. insects, food waste, biofuel by-products), and methods of analysis and sampling. The report and its recommendations considered the particular sources/products, hazards, the potential for transfer to food, the potential impact on human and animal health, and knowledge gaps. Chemical hazards highlighted included, in particular, persistent organic pollutants, natural contaminants, veterinary drugs, and potentially toxic elements. Examples of biological hazards included bacterial pathogens, parasites and prions; whilst physical ones comprised radionuclides, nanomaterials and microplastics. In conclusion, the importance of animal feed safety for animal and consumer health is increasingly being recognized. Internationally harmonized guidelines and best practices for feed safety are essential for protecting animal and human health.

Keywords

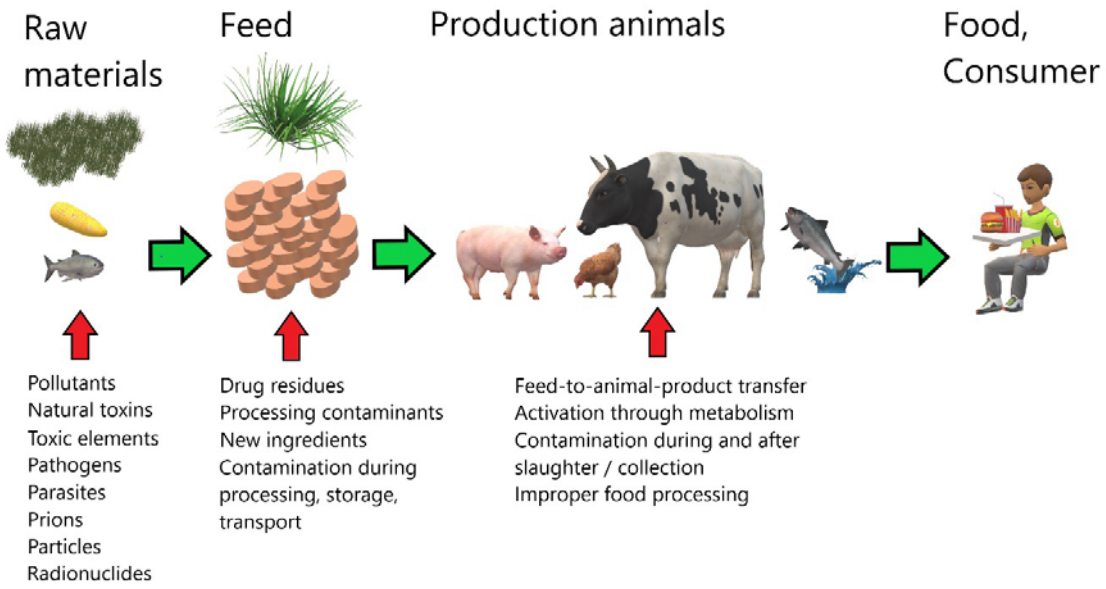
animal feed, food safety, risk assessment, animal health, hazard identification, international harmonization

1. Introduction

Demand for animal feed used in the production of food-producing animals is projected to increase in the near future. This increase in demand relates to the predicted growth of the global population. Within that population, there will also be continually more consumers with sufficient income to buy foods produced by animals. In addition, there is a parallel trend towards increased sustainability of animal feed production. This trend may lead to the increased use of feed ingredients without a history of previous use as animal feed. Example of this are

by-products from food processing and biofuel production. Also, the desire may grow to redirect unconsumed human food as animal feed within so-called "circular" food production systems.

The safety of animal feed is important for the health of the animal that consumes it. The hazards that may occur vary widely in their nature. They include microbes such as bacterial pathogens causing animal disease; chemical substances that may be either acutely or chronically toxic, and physical hazards such as small objects or radioactive isotopes. These hazards may enter the food production chain at different stages of production (Figure 1). Some of these hazards, such as so-called "zoonotic" pathogens that cause disease in both animals and humans, may be transferred from the animal feed to the edible parts of the animal. In some cases, certain chemical substances that are taken up by the animal are metabolized, which makes them less or more toxic. The hazards that are transferred to the animal tissues used as foods are also of concern to the consumer. Such hazards are therefore both feed and food safety issues.



Source: Author's own elaboration.

Figure 1: Possible entry points for hazards related to feed

This paper focuses on emerging risks in animal feed, including risks related to previously unknown hazards or to increased exposure to known ones (for example, from new feed sources or increased prevalence due to a changing climate). Besides the risks to animal health, it considers the possible transfer to edible tissues, eggs, and milk, and subsequent consumer exposure. International harmonization of approaches for identification, evaluation and mitigation of food safety risks is key to prevent potential trade disruption over the safety of these products.

2. The FAO/WHO expert meetings

With the growing demand for animal feed and the introduction of new feed ingredients, it is important to ensure that animal feeds remain safe, in order to protect the health of the animals and the consumer of animal-derived food products. Food safety and animal health are both items that international organizations deal with, such as the Food and Agricultural Organization of the United Nations (FAO), the World Health Organization (WHO), and the International Organization for Animal Health (WOAH). These organizations also work together on animal feed safety. FAO and WHO, for example, convened international meetings with experts who had been asked to provide their views on what hazards should be prioritized and what actions governments and industry should take to uphold the safety of feed. The first of these workshops took place in 2007 (FAO and WHO, 2007). It considered the activities that the various international organizations (e.g. FAO, WHO, WOAH) carried out in the field, the impact on international trade in food and feed, the principles of risk assessment, and a list of relevant chemical and microbiological hazards and methods to detect them. The workshop concluded with a list of recommendations for the establishment of international standards and criteria for the safe use of feed ingredients, methods to decontaminate feed, integration of feed safety into food safety emergency warning systems, communication platforms for exchange on feed safety, and collaboration and communication between governments and industry.

A follow-up workshop was hosted by FAO and WHO in 2015 against the background of the rapid developments in the animal feed sector since the previous workshop in 2007. It considered the various hazards in animal feed that could become a risk for human health after their transfer

from feed to food products of animal origin. Particular attention was paid to the hazards that were likely to emerge as a result of the use of feeds and feed ingredients containing them, and the potential risks associated with certain technologies for feed production.

The recommendations that resulted from this workshop considered five different areas, namely: 1) the need for internationally harmonized standards; 2) the actions that FAO, WHO, WOAH, Member Countries, and industry could undertake to ensure that feeds are safe; 3) the support that FAO, WHO, Codex Alimentarius, the Organisation for Economic Cooperation and Development (OECD), and their respective member countries could give to risk assessment of animal feed; 4) what research the scientific community, member countries, and industry has to carry out, and 5) what actions FAO and WHO have to undertake to produce data, methods and guidelines that will support the risk assessment of animal feeds (FAO and WHO, 2019).

Appended to the meeting report was an extensive background document that explored the different categories of safety hazards (i.e. chemical, biological and physical) in a systematic manner. For the three different hazard categories, the report considered specific hazards and their sources. It also described the strength of the evidence for the potential transfer of the hazards from feed to food from animal origin, and their potential impact on animal and human health. It also considered the knowledge gaps that would require further research into the characteristics, behaviour, and occurrence of a particular hazard. In addition to specific hazards, the report also addressed various types of feed ingredients and feed production technologies that are becoming increasingly important to the sector. Finally, it discussed the specific feed products and the hazards associated with them, and what their potential effects on animal and human health could be, as well as the knowledge gaps.

Notably, a number of the hazards explored were also more concisely featured in another report of FAO and the International Feed Industry Federation (IFIF) published in 2020. It is an updated version of the good practices manual for the feed industry. Besides hazards, it also addresses principles of safety governance, good manufacturing practices, quality assurance, on-farm practices and methods for sampling and analysis, with many practical examples (FAO and IFIF, 2020).

Below, we highlight several examples for each hazard category. For more detailed information, the readers are referred to the actual report (FAO and WHO, 2015).

3. Chemical hazards

Chemical hazards fall into different categories, such as persistent organic pollutants, natural contaminants, veterinary drugs and toxic elements.

Persistent organic pollutants include substances that are very stable and ubiquitously present in the environment at very low levels. Some of these compounds are exclusively man-made, whilst others may originate from both natural and non-natural sources, such as dioxins from clay minerals and waste incineration, respectively. Polychlorinated aromatic compounds, such as dioxins and polychlorinated biphenyls (PCBs)s are well-known hazards from this category. They have the propensity to accumulate throughout the biological food chain because they accumulate in body fat. In animals they accumulate particularly in the liver, where they are also metabolized. Tissues of fatty fish may also contain these compounds and are therefore involved in the feed-to-food transfer. Fishmeal, which is a common feed ingredient for livestock production and aquaculture, is known to be a particular source of these compounds. There is strong evidence for the toxicity of dioxins and PCBs to humans (e.g. Tavakoly Sany *et al.*, 2015), and they are recognized as carcinogens which act through non-genotoxic mechanisms. The data for toxicity to animals is limited.

The report also considered two groups of natural contaminants, namely mycotoxins and plant toxins. Mycotoxins are a very diverse range of toxic compounds with acute and chronic toxicity. They are formed by moulds that grow on crops in the field or post-harvest on the harvested crop or food/feed product if the conditions of moisture and temperature are favourable. A well-known example of a mycotoxin is aflatoxin B1 formed by various strains of *Aspergillus* moulds. It is converted to aflatoxin M1 and transferred from feed to milk in dairy cattle. The range of known mycotoxins grows as researchers make new discoveries. Plant toxins are also very diverse and these can be an issue when plants that contain them are freshly consumed (for example, by cattle grazing on pastures) or are part of processed crop feeds (for example, pelleted feed) or herbs or extracts of such plants added to feed. For many toxins, there is scarce data on the potential for transfer from feed to food.

Veterinary drugs, such as tetracyclines, are part of medicated feeds, which were not the target of the report. Unintended cross-contamination may occur in factories that produce medicated feed. Although the levels of drug residue may be low, risks may occur because such contaminated feeds will typically be fed without knowledge of the contamination. This means that the feed may be given to livestock species that were not the target of the contaminating medication. For example, coccidiostats can be used for poultry, are toxic to horses.

Potentially toxic elements include heavy metals such as cadmium, lead and mercury. These materials are particularly an issue in marine organisms. Fish meal, which is a popular ingredient in feeds for fish, could be a source of methylmercury, for example.

4. Biological hazards

Biological hazards cover different scales, ranging from prions (proteins) via micro-organisms (such as bacteria) to parasites (such as intestinal worms). Amongst the micro-organisms that the report considered were various well-known food pathogens such as *Salmonella, Listeria,* specific strains of *Escherichia coli* (which are known to cause intestinal bleeding), *Clostridium, Brucella* and *Mycobacterium*. The report highlighted a number of features. One of these features was the acquisition of *Salmonella* from contaminated feed. The acquisition of *Salmonella* frequently occurs because the hygienic measures in the production facilities are insufficient, or if infected animals (domestic and wildlife) have access to stored raw materials. Another feature addressed in the report is the resistance against multiple antibiotics in *Mycobacterium*, which may compromise clinical treatment of consumers once they fall ill.

For parasites, there is evidence for the feed-to-animal and food-to-consumer transfers. By contrast, there is little documentation of the transfer from feed to food. Prions are the misfolded forms of naturally occurring proteins. They particularly accumulate in so-called specified "risk materials" such as brain and nerve tissue of infected cattle. Prions are believed to cause "mad cow's disease" in cattle and Creutzfeldt-Jakob disease in humans. The report observed that appropriate measures, such as the prohibition to use specified risky materials as food or feed, can help contain the infection of animals and humans by these prions.

5. Physical hazards

Radionuclides, which are the radioactive isotopes of elements, may enter the food supply in different ways, such as from environmental exposure of food-producing organisms after incidents, leakage from sources, or natural background occurrence. Radioisotopes all have characteristic half-lives, which may vary considerably from one isotope to another. Therefore, risk managers should take the half-life length into account to ensure that the measures that they take against adverse effects are appropriate.

Nanomaterials are materials with distinct structures (such as particles) on the nanometer scale. Their properties and behaviour, and therefore also their toxicological profile, may be completely different from larger particles made from the same materials (De Matteis and Rinaldi, 2018). Scientific literature shows an increasing number of reports on potential uses of nanomaterial-containing feed additives (e.g. Reddy *et al.*, 2020). The report observed that there was still limited information available on the safety of nanomaterials, such as their potential for transfer from feed to food.

Micro- and nanoplastics contain particles in the micro- and nanometer scale, which, in some cases, may also be the result of degradation of larger plastic objects. These plastics may spread through the environment before winding up in feed sources (for example, harvested from contaminated aquatic environments). These plastics may also be an absorbent for contaminants and pathogens that are then transported with the plastics as they move through the environment.

6. Conclusions

The importance of animal feed safety for animal and consumer health is increasingly being recognized (FAO and WHO 2015). It also identified a need for internationally harmonized guidelines for the risk assessment of feeds and best practices (for example, for production). These guidelines and best practices would then complement the activities and outputs from various international organizations already active in the field, such as the WOAH's health codes for both terrestrial and aquatic animals, Codex Alimentarius guidelines on animal-derived foods, and the International Feed Regulators. The experts convened in the meeting also identified and reviewed a number of hazards that have recently started to emerge, and provided new insights on some existing ones as well. While stakeholders increasingly recognize the importance of food safety for food security ("If it's not safe, it's not food"), the same also holds true for animal feed. The efforts of the international organizations towards harmonization of countries' efforts to manage feed safety will therefore ultimately contribute to the maintenance of a safe food supply for future generations.

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Connecting animal movements and other risks to optimize surveillance in West Africa

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1. Presentation summary

West Africa (WA) is characterized by a dynamic livestock subsector. It represents 44 percent of the agriculture gross domestic product (GDP) of the Economic Community of West African States (ECOWAS) region (Leonhardt, 2019), and is the second provider of employment (80 million), of livelihoods, food security and resilience as well as a major contributor to attainment of the Sustainable Development Goals. Pastoralism with periodical transhumance is the dominant production system and is strongly influenced by climate variability and agro-ecological zones. Besides the seasonal search for water and pastures, livestock mobility can be triggered by crises and disasters, leading to population displacement. Mobility has also important socioeconomic and environment benefits, including supplying major livestock markets in coastal areas and major urban centres in West and Central Africa, and sustainable use of resources in drylands.

The prevalent insecurity in vast areas of the Sahel, coupled with low capacity of national animal health systems, limits the health and extension services provided to pastoral herds and the ability to certify the health of animals crossing porous borders on foot. Therefore, the pastoral production system is also associated with substantial health risks and threats. Mobile herds are exposed to diseases and vectors, and can introduce and spread high-impact pathogens, antimicrobial resistant germs and vector-borne infections in countries /zones that had been free of these threats, thus causing devastating sanitary and socioeconomic consequences.

A better understanding of animal flow dynamics in West Africa is key to assessing rigorously the health risks associated with it and to inform risk-based surveillance systems towards effective early detection and mitigation of animal health threats. However, the shortage of data on animal mobility, identification and traceability remains a major challenge.

To address this gap, the Food and Agriculture Organization of the United Nations (FAO) has been championing and strengthening the ability of animal health professionals of ECOWAS member countries to conduct animal mobility studies and map the health risks and threats along the pathways. The main source of data for this study was standardized digital questionnaires and official recordings at crossing border points. Geographic Information System (GIS), descriptive statistics, and social network analysis (SNA) tools were used to process and analyse the data. The GIS tools helped visualize the results of the mobility patten, building on various layers of themes (e.g. administrative boundaries, road and railway networks, water points, markets, slaughterhouses). These tools allow the movement of animals to be displayed as flows which are overlapped with risk factors and SNA indicators. Outputs risk maps are generated and stored on a repository website and accessible to country users to inform surveillance and preventive interventions. For instance, the assessment and mapping of Rift Valley Fever risks, integrating mobility and other risk factors such as density, accessibility, and rainfall, and using a multiple-criteria decision analysis (MCDA) tool helped identify hot spots for the introduction and spread of the disease in Senegal.

Another major output of this work is to highlight the critical importance of a comprehensive value chain (VC) approach to adequately assess health risks and threats, and optimize disease surveillance and risk reduction interventions. These VC studies enable a better understanding of livestock production and marketing systems, interfaces, knowledge, attitudes and practices associated with health threats and risks. In WA, FAO has supported several VC studies and risk assessment, including on (i) poultry and highly pathogenic avian influenza (HPAI)-related risks in Guinea, (ii) bushmeat and Ebola virus disease (EVD) risks in Senegal, and (iii) ruminants and anthrax risks in Guinea. FAO has also developed an Epidemiology Value Chain Platform (EVC) tool to collect field data at animal gathering points and instantly display them for use by veterinary services and other stakeholders. This tool also helps map, characterize and visualize high-risk locations along the VG (especially markets/abattoirs) and movements between them in a dynamic online interface. The EVC tool supports decision-

making, policies, capacities and interventions to mitigate risks at critical points along chains.

In conclusion, FAO has also been strengthening member countries' capabilities on real-time community reporting, lab diagnostic and information system, and preparedness in order to enhance effective risk reduction. This holistic approach to risk assessment and mitigation at country and regional levels is key to understanding drivers of disease spread and to ensuring safe and sustainable mobile livestock production system in the region.

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Detection of antimicrobial residues in meat and milk in Tunisia

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Abstract

Misuse of veterinary drugs generates residues in food of animal origin, particularly in meat and milk. These residues can be responsible for various human health issues. Thus, detection of residues by effective and reliable analytical methods is important. This study aimed to screen antimicrobial residues in 49 samples of meat (bovine, ovine and poultry meat) and 28 samples of cow's milk collected in Tunisia during 2018 and 2019, using the radioimmunoassay Charm II test. Among the 49 meat samples, two poultry samples (4.1 percent) were suspected positive for tetracyclines, six poultry samples (12.25 percent) were suspected positive for sulfonamides and four sheep samples (8.16 percent) were contaminated with macrolides. No contamination with beta-lactams or streptomycin was detected. All cow milk samples were compliant for tetracyclines, sulfa-drugs, macrolides and aminoglycosides, and only two of 28 samples (7.15 percent) were suspected to be positive for beta-lactams. This study's results highlight how important it is to set up effective national food control systems for veterinary drug residues.

Keywords

drug, antimicrobial, residues, antimicrobial resistance (AMR), antimicrobial use (AMU), meat, milk, Charm II, screening.

1. Introduction

The demand for meat as a protein source and milk as an excellent source of calcium has increased considerably around the world. To produce enough meat and milk to meet this growing demand, intensive farming is frequently used. Antimicrobials are increasingly used to treat diseases, but prophylactic and metaprophylactic treatments are also employed to prevent the spread of infections from sick animals to healthy animals in the same production unit (Reig and Toldrá, 2008). In addition, antimicrobials are sometimes used to promote growth (Butaye, Devriese and Haesebrouck, 2003). However, the two possible consequences of antibiotic use in livestock and poultry are the chemical danger and the biological danger occurring in meat and milk (Muehlhoff *et al.*, 2013). Chemical hazards include residues of veterinary drugs that can be found in milk, meat and meat products, especially if the dosage recommendations and/or necessary withdrawal periods are not respected. The biohazard can include medical waste or samples of a microorganism, viruses, or toxins (from a biological source) that can affect human health (Muehlhoff *et al.*, 2013).

Consumption of meat and milk containing excessive amounts of antimicrobial residues poses several risks to human health including immediate toxicities such as allergic reactions or longer-term health problems such as cancer or disturbance of the human microbiota (Paige, Tollefson and Miller, 1999). Antibiotic residues in meat

and milk may also lead to the selection of bacteria with resistance characteristics. In fact, antimicrobial resistant bacteria can represent a reservoir of genes transferable to pathogenic or commensal bacteria in the digestive tract and therefore could be a serious threat to disease treatment in humans and animals (Bamidele Falowo and Festus Akimoladun, 2020). The contribution of food, in particular, meat and milk, as a vector or source in humans of antibiotic resistant bacteria is a major problem for medicine in the 21st century (Fair and Tor, 2014).

The monitoring and control of compliance with the admissible thresholds in foodstuffs for antimicrobial substances, present in the form of residues, are defined by regulations and ensured by official monitoring/surveillance plans. Several tests to detect residues in meat and milk have therefore been developed (Navrátilová, 2009; Shankar *et al.*, 2010). In Tunisia, there are no validated screening methods included in the residue monitoring plan. In this context, the Radiochemistry Laboratory of the National Center for Nuclear Science and Technology uses the Charm II technique, which is a sensitive radioimmunoassay based on the use of radioactive markers for detection of antimicrobial residues in milk as well as bovine, ovine and poultry meat at the European Union recommended limits (MRLs). The Charm II radio receptor assay technique was developed by Charm Sciences Inc. and is based on the use of H-3 and C-14 labelled radiotracer compounds along with a scintillation counter. The analytical process involves competition between the target drug residue in the sample and an amount of ^{3}H or ^{14}C labelled drug (tracer) to bind on microbial cells of the receptor. The amount of tracer that binds to the receptor sites is then measured (Mukota *et al.,* 2020). Samples with high signal are considered negative (the receptor sites are largely bound with the labelled antibiotic) while samples with low counts are considered positive (the receptor sites are bound with the target antibiotic). The Charm II test allows not only the identification of samples with residues, but also specifies to which family of drugs the residue belongs.

In Tunisia, despite the potentially serious effects of antimicrobial residues on human health, there is no published data about the contamination of milk and meat samples with these residues. The objective of this paper was to assess the presence of residues in randomly collected samples of milk and meat marketed in Tunisia by utilizing the Charm II test.

2. Materials and methods

Reagents and standards

The antimicrobial test kits were obtained from Charm Sciences Inc. (Lawrence, United States of America). For analysis of meat samples, Charm II kits of sulfonamides (SMMSU-022C), tetracyclines (TMSU-025), streptomycin (STMSU-023A), macrolides (EMSU-023A) and beta-lactams (PMSU-050A) were used. Milk samples were analysed with kits for the detection of sulfonamides (SULFA-028B), tetracyclines (TET2-041E), aminoglycosides (STTBL-025C and GTBL-020A), macrolides (ETBL-028B) and beta-lactams (TBL8-049C).

Samples

Some of ovine, bovine and poultry meat samples were collected from the Tunisian markets in the period of 2018–2019. Others were as part of the Tunisian residue control plan of 2018 and 2019. A total of 49 meat samples (15 bovine, 20 poultry and 14 ovine) were analysed. Meat samples were taken by carefully removing the excess fat. Most of the samples were assayed immediately and those that were not used immediately were stored at -20 °C for a maximum of two months. Before performing the test, the frozen samples were thawed gently.

The 28 cow milk samples were received in the framework of the national residue control plan during 2018 and 2019. Milk samples were refrigerated and tested within 5 days of milking.

3. Results

Table 1 illustrates the results of the screening for antimicrobial residues. Two out of 49 samples (4.1 percent) were suspected positive for tetracycline residues, six out of 49 samples (12.25 percent) were suspected positive for sulfa-drug residues and 8.16 percent (four of 49 samples) were contaminated with macrolides. All samples were compliant for beta-lactams and streptomycin residues. The samples suspected of containing tetracyclines and sulphonamides were poultry meat, which suggests that these veterinary drugs may have been improperly used on poultry farms to prevent and combat diseases related to intensive farming conditions. Only samples of ovine meat were contaminated with macrolides.

The results obtained for milk samples are summarized in Table 2.

Table 1: suspected positive samples of bovine, ovine and poultry meat in Tunisia

Species	Sulfonamides	Beta-lactams	Macrolides	Streptomycin	Tetracyclines
Bovine	0/15	0/15	0/15	0/15	20/20
Ovine	0/14	0/14	4/14	0/14	20/20
Poultry	6/20	0/20	0/20	0/20	2/20

Source: Authors' own elaboration.

Table 2: Detection of veterinary drug residues in Tunisian cow milk

Antimicrobials	Analysed samples	Non-compliant samples	Non-compliance rate
Tetracyclines	28	0	0
Sulfa-drugs	28	0	0
Macrolides	28	0	0
Beta-lactams	28	2	7.15%
Aminoglycosides	28	0	0

Source: Authors' own elaboration.

We remark that all samples were compliant for tetracyclines, sulfa-drugs, macrolides and aminoglycosides and only two out of 28 (7.15 percent) were suspected positive for beta-lactams. This result suggests that beta-lactams are frequently used in dairy farms and the withdrawal periods are not always respected.

4. Conclusions

This work provides a basis for establishing a database on Tunisian milk and meat contamination with antimicrobials by using the Charm II test. Routine use of these procedures would help food agencies and the government to better regulate the use of antibiotics in poultry and livestock. The described procedures could also be used to correlate the presence of residues in milk and meat with antimicrobial-resistant bacteria in food-producing animals. The Charm II test is a quick, reliable and simple method that could help to consolidate the efforts of the national meat and milk monitoring network and establish a national database on families of antibiotics.

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Validation of veterinary diagnostic tests for infectious diseases: A review of challenges and opportunities

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Abstract

Diagnostic tests for infectious diseases are widely used to inform critical animal health management and trade-related decision making. Thorough validation of these assays is vital to ensure that diagnostic tests are fit for their designated purpose. The World Organization for Animal Health (WOAH) has developed a science-based process for purpose-oriented validation, certification and registration of veterinary diagnostic tests for infectious diseases. The WOAH assay validation pathway includes best practice guidelines for determining critical test performance parameters such as analytical sensitivity and specificity, diagnostic sensitivity and specificity, selection of an appropriate cut-off, repeatability and reproducibility that provide an objective assessment of an assay's fitness for purpose and enable statistically sound test interpretation. Despite international acceptance of this standard, adherence to the WOAH assay development and validation pathways remains a challenge for many stakeholders. The traditional diagnostic test evaluation methods described by the WOAH require a perfect reference assay and access to sufficient numbers of well characterised reference samples, which are commonly reported obstacles to compliance. In many cases, validation studies are poorly or incompletely reported. Meanwhile, increasingly rapid development of "new technologies" such as point-of-care tests, multiplexed and biomarker assays and next generation sequencing technologies present emerging challenges, as the traditional validation framework still needs to be adapted to their methods and outputs. Verification of previously validated tests also continues to present a challenge for diagnostic

laboratories looking to implement new commercial assays or make alterations to existing assays. Whilst such challenges continue to pose a threat, they are also presenting new opportunities. The application of Bayesian Latent Class Modelling and other alternative approaches to test validation science such as network approaches and interlaboratory comparisons may overcome the limitations of traditional validation methods, whilst the application of validation templates, the development of improved reporting standards and the emergence of test certification schemes are facilitating more complete, transparent and objective reporting of validation data. This paper provides examples of challenges and opportunities that test developers and end users may encounter during test development, validation and verification studies.

Keywords

test validation, verification, sensitivity, specificity

1. Introduction

Chapter 1.1.6 in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH Terrestrial Manual) describes principles and methods for the development and validation of diagnostic tests of infectious diseases (WOAH, 2018). Validation is defined as "a process that determines the fitness of an assay, which has been properly developed, optimised and standardised, for an intended purpose." Since its publication in 2013, the WOAH assay validation pathway has acted as the internationally recognised standard for performing diagnostic test validation.

Whilst the WOAH assay development and validation pathways remain an effective roadmap for the design and implementation of new diagnostic assays, compliance with these internationally recognised standards remains a challenge for many stakeholders. These challenges are many and varied and arise at various stages throughout the process. In many cases, the requirements of the standard are deemed too onerous or simply cannot be achieved due to resource limitations. Knowledge gaps remain amongst those performing the process, and access to training in test validation science remains limited. Meanwhile, increasingly rapid development of new technologies such as point-of-care tests, multiplexed and biomarker assays and next generation sequencing technologies present emerging challenges, as the traditional validation framework still needs to be adapted for these methods and outputs.

In addition to challenges with test development and validation, there is increasing focus on issues associated with test verification. Verification is the process of determining that a previously validated assay (such as a commercially available test kit) performs as expected and remains "fit for purpose" prior to implementing the test in a new laboratory or following minor changes in test method. Standardised processes for performing verification studies (including comparability studies) remain limited, and improving the quality and reporting of diagnostic test verification studies presents a significant opportunity to improve transparency and confidence in diagnostic test performance.

In this paper we review the WOAH assay development and validation pathways, discuss the challenges faced by those performing test validation and verification studies, and identify opportunities for improvement.

2. The WOAH assay development and validation pathways

The WOAH assay development and validation pathways describe a standardised framework for performing and reporting validation studies for new diagnostic tests. The process is divided into three sections: assay development pathway, assay validation pathway, and retention of validation status (Figure 1).

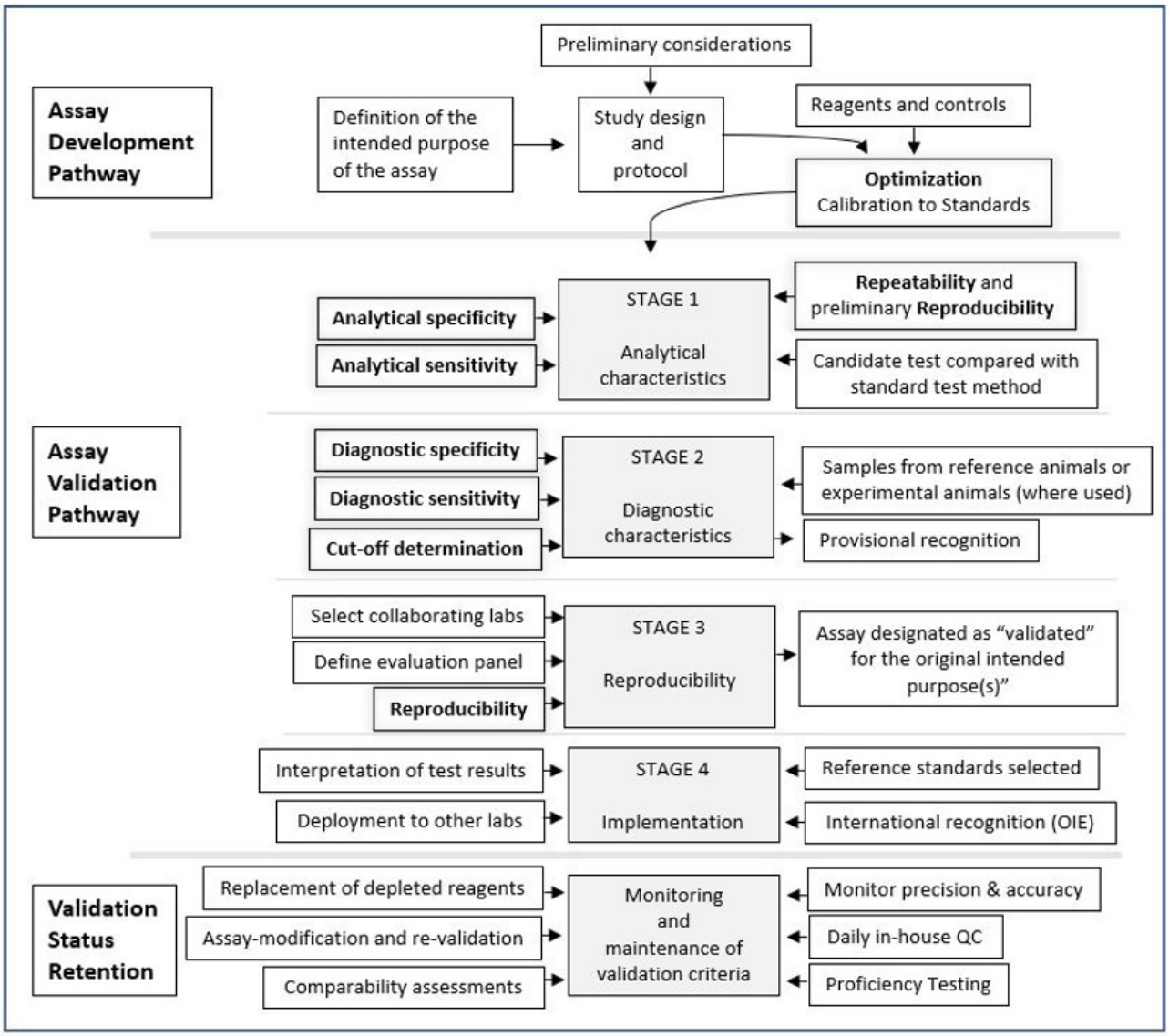
Assay development pathway

In the initial assay development phase, the purpose of the test must be well defined. The definition should consider the target populations in which the test is to be used (both in terms of geographical location and species), the sample type (such as serum, plasma or milk), as well as the desired performance characteristics (for example, a screening assay designed to detect, with high sensitivity, all animals infected with a specific pathogen in a population of a given host species in a defined geographical location) (WOAH, 2018).

The purpose of the test will significantly influence the design of the validation study, from the initial protocol development through to selection of appropriate reference samples and determination of a fit-for-purpose cut-off value. Table 1 illustrates seven WOAH test purposes and emphasises relevant diagnostic performance parameters such as diagnostic sensitivity and specificity (DSe, DSp), positive and negative predictive values (PV+, PV-) and likelihood ratios (LR+, LR-) for testing of historical disease freedom, re-establishment of freedom after a disease outbreak, international trade and movement, eradication, confirmatory testing of clinical cases, prevalence estimates and determination of immune status.

A test can be designated fit for more than one purpose. Under these circumstances, validation data are required to support each purpose separately.

With the purpose defined, the test can be designed accordingly. In addition to being "fit for purpose", assay characteristics which should be considered include QA/QC capability, throughput, turn-around, automation, costs, maintenance, operational skills and acceptance in the scientific and regulatory communities.



Source: Chapter 1.1.6 Principles and methods of validation of diagnostic assays for infectious diseases, WOAH Terrestrial Manual 2021. Modified version.

Figure 1: Assay development and validation pathways with assay validation criteria highlighted in bold typescript within boxes

Table 1: Test purposes and relative importance of diagnostic sensitivity (DSe), diagnostic specificity (DSp), positive predicitive value (PV+), negative predictive value (PV-), likelihood ratio of a positive test result (LR+) and likelihood ratio of a negative test result (LR-)

Purpose	Example and relevant parameters
1a) Historical freedom (with or without vaccination)	In a population which is historically free of a particular disease/pathogen the prevalence is zero (or close to zero). To be fit for purpose the test or test algorithm aims to minimise chances of false-positive results and ideally requires **high DSp**, **high PV+ and high LR+**. This can be achieved by a single test with a high DSp or serial testing (*).
1b) Re-establishment of freedom after outbreak	During the course of a successful disease control programme, a gradual shift in prevalence from high (during the peak of the outbreak) to low (at the tail end of an outbreak) can be expected. During the early stages of a proof-of freedom testing programme, when disease prevalence remains at non-negligible levels, a fit for purpose test needs a **high DSe**, **high PV- and high LR-**. This approach minimises the chances of false-negative results and allows detection of positive individuals. This can be achieved by a single test with a high DSe or parallel testing (*). At the end of the disease control programme, when the remaining infected animals have been removed from the population, disease prevalence will be very low and so the proof-of-freedom testing algorithm will likely need to be altered to increase DSp (and thus improve PV+ and LR+).
2) Certify freedom from infection or agent in individual animals or products for trade/ movement purposes	For the purpose of trade and movement the probability of false-negative results needs to be minimised. Otherwise, infected animals could be traded or moved with the potential to spread infection into non-infected, healthy populations. As the test is applied on individuals, no or little information is available about the prevalence or pre-test probability of infection. To be fit for purpose the test or test algorithm aims to minimise chances of false-negative results and ideally requires **high DSe**, **high PV- and high LR-**. This can be achieved by a single test with a high DSe or parallel testing (*).
3) Contribute to the eradication of disease or elimination of infection from defined populations	This purpose follows a similar pattern as in 1b, where prevalence is expected to decrease from high to low over time in a defined population.
4) Confirm diagnosis of suspect or clinical cases (includes confirmation of positive screening test)	The goal of a confirmatory test is to minimise the chances of a false-positive result. <u>Confirmation of clinical cases</u> For the purpose of confirmation of a clinical case ideally a test with a **high DSp**, **high PV+** and **high LR+** is needed. Because of the clinical manifestation of the disease and expected high pathogen load DSe is not considered to be as relevant. <u>Confirmation of positive screening samples</u> Screening tests are applied on healthy populations. They usually have a high DSe to ensure infected individuals are not missed. Only if confirmed by a confirmatory test with a high DSp the animal is considered positive. In this case the confirmatory test* needs to have a **high DSp**, **high PV+** and **high LR+**. This approach follows the series testing algorithm (*).
5) Estimate prevalence of infection or exposure to facilitate risk analysis	Epidemiologists require reliable estimates for test accuracy to design sampling plans for prevalence studies, surveys, herd health status and disease control measures. Using a screening test with a high DSe followed by a confirmatory test with a high DSp is a common approach for prevalence estimations.
6) Determine immune status a) in individual animals post-vaccination b) estimate sero-prevalence post-vaccination (research and monitoring of vaccine efficacy)	For this group the aim is to have a **high DSp**, **PV+** and **LR+**. A false-positive result could have fatal consequences because such an animal could in fact not be vaccinated/ protected. The higher the accuracy of the test the more precise will be the estimate of post-vaccine seroconversion in individuals and populations. An example is the Fluorescent Antibody Virus Neutralisation (FAVN) test to assess the immune status of dogs and cats post-vaccination against rabies virus. For international travel a result of > 0.5 IU/ml is considered to represent acceptable protection.
7) Other	This is an open purpose that can be defined by the test developer.

Source: Authors' own elaboration.

Notes: a (*) Multiple testing.

Multiple testing consists of using more than one test to determine the infection status of an animal. The most common algorithms are testing in series or in parallel.

For example, if two tests are used in series a sample is considered positive only if the first and the second test are positive. Series testing increases DSp but decreases DSe and increases PV+ and LR+.

Confirmatory testing follows the series testing approach because a positive result from a screening test (high DSe) needs to be confirmed by a second test with a high DSp. The confirmatory test needs to have at least the same DSe as the screening test otherwise they could generate false-negative results, which would be considered a true-negative in this algorithm. Confirmatory testing increases DSp but decreases DSe and increases PV+ and LR+.

If two tests are used in parallel a sample is considered positive if any of the tests or both tests are positive. Parallel testing increases DSe but decreases DSp and increases PV- and LR-.

In addition, for multiple testing algorithms to be effective screening and confirmatory tests should not be conditionally dependent (for example measure the same analyte, such as two ELISAs using the same antigen). Positive dependence in test sensitivity reduces the sensitivity of parallel test interpretation and a positive dependence in test specificity reduces the specificity of serial interpretation (Gardner *et al.*, 2000).

Assay validation pathway

The assay validation pathway follows the assay development pathway and is divided into four stages: analytical characteristics (including repeatability), diagnostic characteristics, reproducibility and implementation. In the analytical and diagnostic characteristic stages critical test performance parameters are estimated, including:

Analytical sensitivity

Analytical sensitivity (ASe) is a measure of the technical ability of an assay to detect replicates of increasingly small concentrations of analyte (e.g. antibodies) in a sample. Analytical sensitivity is determined by testing serial dilutions of known positive reference samples to determine the limit of detection. For molecular tests, the lowest template copy number that can reliably be detected defines the limit of detection (LOD). When the results for the prototype assay are compared with those from other established assays, a relative measure of ASe can be estimated, and ideally this should be comparable to or better than the reference test (Bowden, Crowther and Wang, 2021). For examples, see evaluations of the Hendra virus (HeV) indirect and blocking antibody detection ELISAs as compared to virus neutralisation (Colling *et al.*, 2018) assays. Note that ASe is distinct from DSe (see below).

Analytical specificity

Analytical specificity (ASp) refers to the ability of a test to distinguish between the target analyte and similar yet distinct analytes. Analytical specificity is assessed by testing reference samples known to be negative for the diagnostic target of interest, but positive for closely related analytes. Assessing the analytical specificity of a new molecular real-time polymerase chain reaction (RT-PCR) assay for the detection of an exotic viral pathogen may involve testing samples known to be positive for closely related or clinically similar endemic viruses, to assess for the presence of "cross-reactivity"; for example, assessing the analytical specificity of an Foot-and-Mouth Disease PCR could be performed by testing reference samples known to be positive for vesicular stomatitis virus, swine vesicular disease and/or malignant catarrhal fever. Analytical specificity assessments cannot determine the full range of potential cross-reacting analytes present in the population, or account for population-level sample variability; as such, determining the analytical specificity is not a surrogate for assessment of diagnostic specificity (see below) (Saah, 1997; Bowden, Crowther and Wang, 2021).

Repeatability

Repeatability is the ability of an assay to produce precise results when the procedure is repeated in replicate. High repeatability is a desirable test characteristic and describes assays that produce minimal variation in results between replicates of both positive (including high and low/threshold positive) and negative samples. Note that when assessing repeatability, the entire diagnostic workflow should be replicated from the point of initial sample processing, to ensure that any variability introduced throughout the procedure is captured (WOAH, 2018; Waugh, 2021).

Cut off, Diagnostic sensitivity (DSe) and Diagnostic specificity (DSp)

Many diagnostic test modalities inherently produce results on a continuous quantitative scale. For example, an indirect enzyme-linked immunosorbent assay (ELISA) designed for the detection of specific antibodies may produce Optical Density (OD) values ranging from 0.0 to 2.0. However, this is determined by the linear dynamic range of the assay, which is dependent on the substrate used, and should be established during the initial development, optimisation, and standardisation of the assay. For the purposes of diagnostic test interpretation, this continuous output variable must be converted to a dichotomous test result (i.e. "Positive" or "Negative"). To achieve this, a "cut-off" value must be selected; all samples returning an output value of below the cut-off are then classified as "Negative", whilst all samples with a test value above the cut-off are classified as "Positive" (Gardner and Greiner, 2006).

However, the distribution of results obtained from a truly negative population will generally overlap slightly with the distribution of results obtained from a truly infected population. In this scenario, there is no possible cut-off value that will correctly classify all individuals in the population; a small proportion of non-infected animals will inevitably be classified as positive (i.e. false-positives) and a small proportion of infected animals will inevitably be classified as negative (i.e. false-negatives). The cut-off value selected will ultimately determine the relative proportion of false-positive and false-negative results (Gardner and Greiner, 2006).

The accuracy by which an assay classifies positive and negative individuals is described by the diagnostic sensitivity and diagnostic specificity.

Diagnostic sensitivity is a measure of how accurately a test classifies truly infected animals. A highly sensitive test will return positive results for a high proportion of truly positive animals, and therefore return few false-negative results (WOAH, 2018).

Diagnostic specificity is a measure of how accurately a test classifies truly non-infected animals. A highly specific test will return negative results for a high proportion of non-infected animals, and therefore return few false-positive results (WOAH, 2018).

Determination of diagnostic sensitivity and diagnostic specificity is a critical step in the WOAH assay validation pathway and is generally performed with the use of 2 × 2 tables, to compare the results obtained from a reference

population of animals known to be infected (based on experimental infection or by a reference test), to results from a population of animals known to be uninfected (Table 2). Diagnostic sensitivity is calculated as the proportion of truly infected animals that test positive in the candidate assay, whilst diagnostic specificity is calculated as the proportion of truly non-infected animals that test negative in the candidate assay (WOAH, 2018). Changes in cut-off entail changes in DSe and DSp (Figure 2a, Figure 2b and Figure 2c).

Selection of a cut-off value

When selecting an appropriate cut-off, the diagnostician must consider the purpose of the test (as defined at the beginning of the validation process). Whilst the most accurate cut-off will be the value at which the total number of false-positive and false-negative results is minimised, the investigator may select a higher or lower cut-off value in situations where diagnostic sensitivity or diagnostic specificity is prioritised (Greiner, Pfeiffer and Smith, 2000; Gardner and Greiner, 2006) (Figure 2a, Figure 2b and Figure 2c). For example, when performing proof-of-freedom testing in populations with low to very low disease prevalence, near-perfect diagnostic specificity is required to improve the predictive value of positive results (see PVs below). In this instance, a higher cut-off value may be selected, at the expense of a slightly decreased diagnostic sensitivity.

Alternatively, pre-border quarantine testing requires assays with high diagnostic sensitivity, as the implications of false-negative results (accidental introduction of a previously exotic agent) are potentially devastating. In this scenario, a lower cut-off value may be selected to reduce the number of false-negative results, while acknowledging that the associated decrease in specificity may result in some truly uninfected animals being denied entry to the country due to false-positive results.

A receiver operating characteristic (ROC) curve, which charts the effect of varying the cut-off value on the diagnostic sensitivity and specificity of the assay, is the preferred method to select a cut-off (Greiner, Pfeiffer and Smith, 2000; Gardner and Greiner, 2006).

Test operators may want to determine an inconclusive or indeterminate range – usually close to the cut-off – and establish a test algorithm to follow up on samples that fall within this range. This usually consists of retesting of samples, for example in the same test and/or another test and/or resampling and retesting of the same animal or animals from the affected cohort (see also sections 3 below for result interpretation).

Reproducibility

Once the analytical and diagnostic characteristics of the assay have been determined in Stage 2, the consistency of performance of the assay in various laboratories should be assessed (Waugh and Clark, 2021). The purpose of reproducibility is to ascertain whether small changes in experimental conditions have a significant impact on assay performance. This is achieved by analysing identical test samples in various laboratories and performing a comparability assessment. A review of factors affecting reproducibility among laboratories is available in Waugh and Clark (2021).

Interpretation of results – predictive values and likelihood ratios

Predictive values are measures of test performance that can be useful for the interpretation of a diagnostic test result. The positive predictive value (PV+) is the probability (%) that an animal with a positive test result is in fact positive/infected.

The negative predictive value (PV-) is the probability (%) that an animal with a negative test result is in fact negative/not infected.

Predictive values can be calculated from 2 × 2 tables as shown in Table 2 (Greiner and Gardner, 2000). Assessment of predictive values highlights the diagnostic uncertainty that exists in certain epidemiological scenarios. This is particularly true when a test with imperfect specificity is used when disease prevalence is low. In this situation, assessment of the PV+ will demonstrate that animals with a positive test result may still be significantly more likely to be non-infected than infected.

Whilst predictive values can be a useful tool in diagnostic test interpretation, they are highly dependent on the prevalence of disease in the population. Predictive values calculated in populations with high prevalence, or at the peak of an outbreak where disease prevalence is high, are not applicable in populations with low prevalence or at the tail end of an outbreak where disease prevalence is markedly decreased. In this scenario, likelihood ratios can be used to assist in test interpretation.

The likelihood ratio is an inherent characteristic of the test; it depends solely on the combined diagnostic sensitivity and diagnostic specificity and therefore does not vary with prevalence (Greiner and Gardner, 2000; Gardner and Greiner, 2006; Caraguel and Colling, 2021).

$$LR(+ve) = \frac{DSe}{1 - DSp}$$

$$LR(-ve) = \frac{1 - DSe}{DSp}$$

Likelihood ratios are extremely powerful, as they can be used to calculate the "post-test" probability of disease, given:

- the observed quantitative test result; and

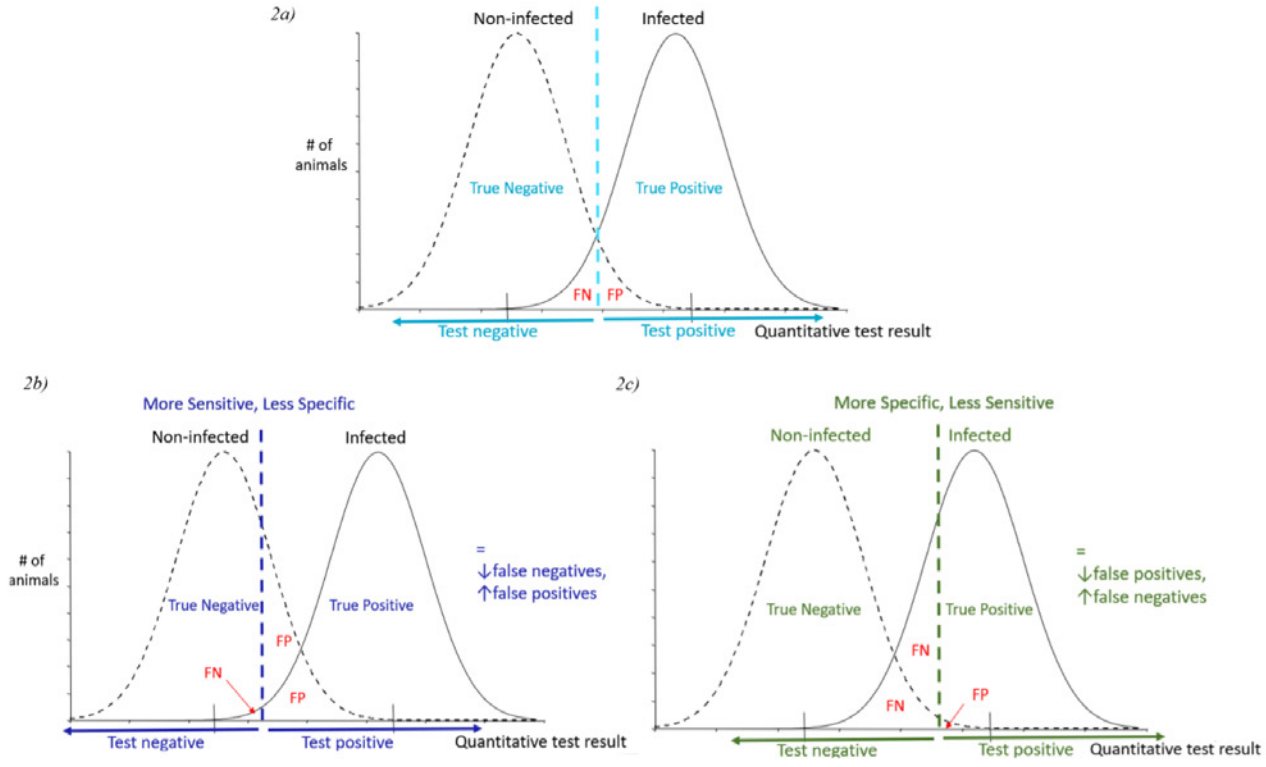
Table 2: 2 × 2 table used to calculate diagnostic sensitivity and specificity and predictive values of a test*

		"True" status		
		Infected	Not infected	
Test Result	Positive	True Positive (TP)	False Positive (FP)	$\frac{TP}{TP + FP}$ = Positive Predictive Value
	Negative	False Negative (FN)	True Negative (TN)	$\frac{TN}{TN+FN}$ = Negative Predictive Value
		$\frac{TP}{TP + FN}$ = Diagnostic Sensitivity	$\frac{TN}{FP + TN}$ = Diagnostic Specificity	

Sources: a WOAH. 2018. Principles and methods of validation of diagnostic assays for infectious diseases (Chapter 1.1.6). *In* OIE – World Organisation for Animal Health, ed. *Manual of diagnostic tests and vaccines for terrestrial animals*. 8th edition, p. Paris, OIE. www.woah.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf

b Gardner, I.A., Colling, A. & Greiner, M. 2019. Design, statistical analysis and reporting standards for test accuracy studies for infectious diseases in animals: Progress, challenges and recommendations. *Preventive Veterinary Medicine*, 162: 46–55. https://doi.org/10.1016/j.prevetmed.2018.10.023

Notes: a Calculation of PV+ and PV- using this method is only valid if the sample population is representative of the target population.



Source: Author's own elaboration.

Figure 2: Changes of cut-off and impact on DSp and DSe, and false-positive and false-negative results

- the diagnostician's assessment of the probability of infection, prior to the test being performed (Gardner and Greiner, 2006; Caraguel and Colling, 2021).

Once the test has been implemented (Stage 4 of the WOAH Assay Validation Pathway), for example in a control or eradication campaign, it is critical that its validation status is retained over time. Replacement of depleted reagents and assay modifications (Figure 1) have the potential to impact test performance, which must be assessed with comparability studies (WOAH, 2019e, WOAH, 2019h; Reising *et al.*, 2021). Ongoing internal and external quality control assists in monitoring accuracy and precision, and are a requirement of accredited laboratories under the International Organization for Standardisation/International Electrotechnical Commission standard ISO/IEC 17025 "General requirements for the competence of testing and calibration laboratories" (WOAH, 2019f; Ludi *et al.*, 2021; Watson, Carlile and Williams, 2021; Waugh and Clark, 2021, Johnson and Cabuang, 2021).

3. Challenges

Whilst the WOAH pathway provides a clear standard approach for the validation of new diagnostic assays, compliance remains a challenge for those attempting to validate new assays.

Defining purpose

Failure to define the purpose of testing will likely lead to errors in both the design of the assay and in the determination of critical test parameters. Such errors may occur when, for example, an inappropriate reference population is selected that is heterologous to the population for which the test is being developed. Such errors have the potential to invalidate the entire assay development process and ultimately result in a test that does not meet the user's needs (WOAH, 2018; Gardner *et al.*, 2021; Table 1).

Reference samples

The WOAH assay validation pathway requires that tests be validated against a specified number of well characterised reference samples. Table 1 in Chapter 1.1.6 (WOAH, 2018) provides the theoretical number of samples from animals of known infection status required for establishing DSe and DSp estimates depending on the likely values of DSe and DSp, and the desired error margin and confidence. Assuming a test with a DSe of 92 percent, DSp of 94 percent and allowing for an error of 2 percent with 95 percent confidence, 707 infected and 542 non-infected animals would need to be tested to establish statistically robust estimates of DSe and DSp for this test.

One of the major challenges of diagnostic test validation is the identification and collection of a suitable number of appropriate reference samples. To obtain realistic estimates for DSe and DSp, reference samples must be selected and classified based on a clear and consistent case definition, be well characterised and be sourced from a population applicable to the defined purpose of the test (Gardner *et al.*, 2021). The infection status can be determined by a) result(s) of one or more reference tests; and/or b) clinical signs and/or; c) experimental infection and/or; d) knowledge about historical freedom or endemism of the disease.

Furthermore, the source population from which reference samples were obtained must be comparable to the target population where the test will be applied. If individuals from the source population had all been in an advanced stage of infection and exhibited clinical signs while the target population where the test will be applied consists mainly of animals in an early or late stage of infection, the test's diagnostic sensitivity is likely to be compromised. Similarly, diagnostic specificity may be affected when a test has been validated in an artificially "clean" source population, for example by using specific pathogen free (SPF) animals, and is then used in the field on individuals that are exposed to a variety of cross-reacting pathogens or other non-specific organisms (WOAH, 2018).

Where access to suitable reference samples from the field is limited, samples generated through experimental infection studies can be used and can provide proof of principle during feasibility studies. However, due to

- differences in selection and dose of inoculum,
- route and site of application,
- selection of organ and tissue types that support infection (predilection sites),
- routes of pathogen shedding,
- kinetics and timing of the pathogen replication cycle,
- nature and magnitude of immune responses,
- presence of related pathogens within the population, and
- animal ethics considerations,

experimentally derived samples are unlikely to be representative of the field-collected samples on which a prospective diagnostic test is likely to be applied. As such, their use as reference samples in a validation study requires careful considerations.

Lack of statistically robust number of samples from infected and non-infected animals is a frequent constraint to satisfying requirements for establishing estimates of DSe and DSp in Stage 2 of the WOAH Validation Pathway (Gardner *et al.*, 2000; Gardner *et al.*, 2021). In these situations, tests with acceptable ASe and ASp, repeatability and promising preliminary DSe and DSp can be provisionally recognised until results from further testing confirm accuracy and overall fitness-for-purpose (WOAH, 2018).

Reference tests

The WOAH assay validation pathway outlines a frequentist approach to diagnostic test validation studies. When assessing the test performance characteristics of DSe and DSp, the frequentist approach makes a critical assumption that all reference samples are correctly classified as truly positive or truly negative samples. In many cases, reference samples are classified (at least in part) based on results from an existing, "gold standard" reference assay (i.e. samples testing positive in the reference assay are determined to be true positive samples).

Similarly, when determining the ASe and ASp, the WOAH pathway compares a prospective assay against an established reference assay which is assumed to have perfect analytical performance.

Whilst a frequentist approach works well in situations where a definitive, highly accurate reference test exists (Heuer and Stevenson, 2021), there are many situations where no such assay is available. The WOAH supports the use of BLCMs and an increasing number of WOAH listed tests have been validated using this approach. Nevertheless, because these statistical models are complex and require critical assumptions, statistical assistance should be sought

to help guide the analysis and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature (WOAH, 2019e). Workshops and training courses are provided by WOAH reference laboratories.

Resource limitations

Performance of diagnostic test validation and verification studies is an onerous time- and resource-intensive activity that requires careful planning and adequate resourcing to enable successful completion. Despite high-economic and social consequence decisions being made on the basis of diagnostic test results, recognition of the importance of diagnostic test validation in ensuring confidence in assay performance is often lacking. This, in turn, leads to inadequate resourcing of laboratories to perform thorough validation and verification studies. The WOAH reference laboratories have an important role to play in facilitating such studies (both internally and in their satellite laboratories) through evaluation and definition of reference methods, data sharing, training, provision of well characterised control material and organization of interlaboratory comparisons (Brown *et al.*, 2021).

Reporting of validation studies

Following a validation study, many novel diagnostic test methods are published and adopted for use in new laboratories or made available for purchase as commercial kits. Communication of diagnostic test validation studies is vitally important to ensure that eventual end users of the assays can confidently select assays that are fit-for-purpose and can verify the claimed assay characteristics in their own laboratories through comparability studies.

Unfortunately, the design of validation studies is often inadequate, and reporting of performance characteristics is not always complete. Many commercial diagnostic kits are provided with limited or inappropriate validation data. In many cases, kit inserts will detail only the results obtained from a few vaccinated or experimentally infected animals, which does not compare to a robust diagnostic sensitivity study with several hundred infected individuals (Crowther *et al.*, 2006). The lack of completeness and transparency is of particular concern where kits are made available on the commercial market and are potentially adopted for field use, where their results may be used to guide important decision making. These issues may, in part, be attributed to the lack of clear standards for reporting of diagnostic validation studies in animals (Kostoulas *et al.*, 2021) and training in their application.

New technologies

The validation of new, emerging diagnostic technologies presents a dynamic challenge for test developers by virtue of their novelty, which can lead to barriers to their adoption in a diagnostic setting. Technologies such as point of care tests (POCT) (Bath *et al.*, 2020; Hobbs *et al.*, 2021), multiplexed formats (Foord *et al.*, 2014), biomarker miRNA (Tribolet *et al.*, 2020) assays and next-generation sequencing (NGS) (Van Borm *et al.*, 2016) all need to be validated for the species, target population and specific sample type that they will be used for. Although the principles of test validation can be universally applied, the current WOAH assay validation pathway cannot be easily adapted to many novel diagnostic approaches.

There is continued discussion about how to properly validate the ever-changing repertoire of new and unique diagnostic reagents, coupled with many novel assay platforms and protocols (Halpin *et al.*, 2021). For example, robustness and ruggedness are considered fundamentally relevant for POCTs and their performance should not be easily affected by fluctuations in temperature, humidity, sunlight, or other environmental factors. The proficiency of independent operators is likely to have a wider variability than that of a team of trained diagnosticians working in an accredited laboratory. There are also jurisdictional regulations for test approval and hierarchy of results with decisions and consequences.

The utilisation of NGS and the number of opportunities for its application to diagnosis are growing rapidly; the major purposes are unbiased sequencing for pathogen discovery and targeted sequencing for detection and further characterisation. If the assay is used for detecting previously unidentified microorganisms, such as during an outbreak investigation, then the primary purpose is diagnostic. If the assay is used to further characterise a previously identified pathogen or to follow the molecular epidemiology of the pathogen during an outbreak, then its general purpose can be described as an adjunct test (Van Borm *et al.*, 2016; Halpin *et al.*, 2021). In the absence of a known target analyte, following a traditional validation pathway is not possible. Monitoring quality metrics such as depth of coverage, uniformity of coverage, GC bias, base-call quality scores, decline in signal intensity or read-length, mapping quality and the inclusion of internal controls are used to assess relative performance of various NGS assays.

Another novel technology that is undergoing rapid progress is the detection of new and novel biomarkers in patient samples, such as micro-RNAs (miRNAs), proteomes or metabolomes. The potential use of these molecules as diagnostic biomarkers is enhanced by their presence and stability in a wide range of sample matrices, including blood, urine, and milk. A thorough review and the constraints and opportunities are presented in Halpin *et al.* (2021).

Another complexity in diagnostic test validation is the development of multiplexed assays such as Luminex and multiplexed real-time RT-PCR/PCR where more

than one target is identified. Test accuracy of each of those targets in different concentrations and distribution is important and challenging to validate. Examples for validation of multiplexed assays are provided by Foord *et al.* (2014) and Perez *et al.* (2020).

Validating tests for wildlife and new emerging diseases

With the continued application of One Health approaches to disease control and the recognition of the role of wildlife in disease emergence, there is increasing interest in the application of diagnostic tests to wildlife populations. Validating diagnostic tests in wildlife populations poses significant challenges, particularly in relation to accessing sufficient number of well characterised, representative samples of adequate quality. Limited knowledge of the infection status of populations, and of host–pathogen biology, pathogenesis, epidemiology, and population dynamics of naturally occurring infections are also frequent limitations (Jia *et al.*, 2020). A thorough review is presented in Michel *et al.* (2021).

The use of diagnostic tests that have been validated for closely related domestic species in wildlife should be approached with caution. Frequently there are flaws in the original test validation studies with incomplete reporting of relevant animal- and population-level information. Even for previously well validated assays, additional validation using target species samples is required to ensure the assay is fit-for-purpose. Where insufficient target-species reference samples are available, provisional recognition of a test for use in wildlife may be possible under the WOAH pathway (WOAH, 2019g; Jia *et al.*, 2020; Michel *et al.*, 2021).

New emerging diseases, particularly zoonoses, present a similar challenge for test validation. Reference tests are unlikely to be validated, and collection and analysis of a sufficient number of well characterised reference samples can be difficult or impossible in the early stages of outbreaks where case numbers are low. This limitation also applies to the emergence of new mutants, new strains and quasi-species (Stevenson and Halpin, 2021).

Result interpretation and communication

The importance of diagnostic test validation and verification in describing the uncertainty associated with individual test results, and how these impact on a test's fitness-for-purpose and result interpretation, is still gaining recognition. Understanding, quantifying and communicating this uncertainty and how it influences the distinction between a "test result" and "diagnoses" remains a challenge for diagnosticians. Utilisation of predictive values and likelihood ratios – generated through validation exercises – for this purpose is a recognised opportunity for improvement (Caraguel and Colling, 2021).

Data management

An additional challenge for validation science is the long-term storage of data, review of validation data and on-going verification that assays are still performing with acceptable diagnostic accuracy.

Laboratory information management systems are required that integrate validation and diagnostic data to provide regular updates of assay performance. At a minimum, data management systems should facilitate:

- storage of validation data in central locations;
- review and/or sharing of data when, for example, assays are deployed to external laboratories (Stage 4 of WOAH Assay Validation Pathway; see Figure 1);
- on-going accrual and integration of diagnostic results to update the diagnostic characteristics of an assay (Stage 3; see Figure 1); and
- storage of Internal Quality Control (IQC) and External Quality Control (EQC) data for use in reviewing assay sensitivity (Stage 1; see Figure 1), especially when changes to reagents or equipment occur.

Access and utilisation of laboratory information management systems (LIMS) for the purposes of test validation and ongoing verification remains limited, as they require significant investment and Information Technology expertise to maintain.

Operator experience and knowledge

Performing and reporting of validation and verification studies requires high levels of technical knowledge in assay development, experimental design, epidemiology, pathophysiology, virology/bacteriology, statistical methods and scientific communication. Lack of appropriate training and guidance in this broad range of fields may lead to errors in the planning, execution and communication of validation and verification activities. As mentioned above, WOAH reference laboratories have a crucial role to play in provision of training and expert consultancy (Brown *et al.*, 2021).

4. Opportunities

Undoubtedly, the obstacles to achieving scientifically robust test validation and verification studies are significant and pose a continuing threat to the accurate use and interpretation of diagnostic tests. However, recognising these challenges aids in identifying opportunities for improvement.

Alternative methods

Bayesian Latent Class Modelling (BLCM)

Bayesian statistical methodologies are being increasingly applied in test validation studies to overcome the constraints faced by traditional diagnostic test evaluation

methods (WOAH, 2018; WOAH, 2019e; Johnson, Jones and Gardner, 2019; Cheung *et al.*, 2021), which require both a perfect reference test and sufficient numbers of appropriate reference samples (Heuer and Stevenson, 2021). Frequentist approaches (as outlined in the WOAH validation pathway), that assume the result of the reference test to be the "true" status of the sample, cannot be accurately applied in cases where there is no "perfect" or "gold standard" reference tests. If an imperfect test is used as a reference test, then the occurrence of "false positive" and "false negative" results will bias the assessment of the new assay.

Bayesian latent class modelling (BLCM) accommodates for this error by simultaneously evaluating the performance of two or more assays using probabilistic models, with the basic assumption that the disease status is latent or unobserved. Both frequentist latent class analysis (LCA) and Bayesian LCAs can be used. While the frequentist LCAs use only the data derived from the samples collected from the population to estimate the diagnostic accuracy measures of the tests, the Bayesian LCAs employ prior information on the disease/condition, diagnostic test performance such as expert opinion or estimates from previous research study in additional to the empirical data obtained from samples collected for the study.

To deploy a traditional LCA model, three key assumptions must be satisfied (Hui and Walter, 1980):

- when multiple populations are being compared, each population prevalence should be different;
- the DSe and DSp of the test are the same across test populations; and
- the tests are conditionally independent.

However, if conditional dependence exists between the index and reference tests (e.g. they both measure a similar analyte), latent class models (LCM) with different dependence structure have been developed to model the conditional dependence among tests.

Discussion of the specific methods for applying BLCM in diagnostic test validation and verification is outside the scope of this paper, but has been reviewed elsewhere (Broemeling, 2011a; Broemeling, 2011b; Cheung *et al.*, 2021).

Collaborative approaches – Network validation and interlaboratory comparison studies

Other emerging approaches to overcoming limitations relating to sample availability, resources and expertise are network validation and interlaboratory comparison studies.

"Network validation" is a system in which numerous laboratories cooperate to generate validation data, which is collated to form a single validation dossier. An example of a collaborative approach to test validation is that of the Australian network of state-based veterinary diagnostic laboratories, the Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR) network. The LEADDR network provides a forum where jurisdictional laboratories can converse with peer laboratories for discussion of assay development and provide practical assistance in the accrual of validation data, especially for Stage 3 (diagnostic characteristics) and Stage 4 (reproducibility) studies. Such an approach has been used to achieve approval and accreditation of a Hendra virus antibody ELISA in the face of a low number of positive reference samples (Colling *et al.*, 2018). Other projects, such as the FAO/IAEA "Vetlab" and "Zodiac" programmes, are adopting a similar collaborative approach to diagnostic validation, verification and capability development (VETLAB, 2019; ZODIAC, 2020). As discussed previously, WOAH Reference Laboratories also play a role in assisting other laboratories with validation and verification activities (Brown *et al.*, 2021).

Network collaborations are also important in performing interlaboratory comparisons. Testing of panels of reference samples in various laboratories can assist in generating information about accuracy and precision of a test, contribute to reproducibility assessments, establish confidence in results and reduce costs and requirements for individual laboratories (WOAH, 2018; WOAH, 2019f; Ludi *et al.*, 2021; Watson, Carlile and Williams, 2021; Waugh and Clark, 2021; Johnson and Cabuang, 2021).

Training, documentation and support

As discussed above, one of the major obstacles to improving the quality of test validation and verification studies is the limited availability of reference samples, expertise and lack of guidance and support for inexperienced operators. Provision of additional training opportunities and guidance documentation may lead to an increase in the quality and reporting of verification and validation studies.

Training and support

ISO/IEC 17025:2017 "General requirements for the competence of testing and calibration laboratories" specifies that personnel must be authorised to perform validation and related activities, which means that training in validation and verification methods, including result interpretation, is likely to become more important to prove competency (Newberry and Colling, 2021). Although free software is available, more training is needed in the design of prospective validation studies, and analysis and interpretation of data. Traditionally FAO/IAEA and the WOAH Reference Laboratories and Collaborating Centres for Diagnostic Test Validation have provided training in these areas and should continue to do so. These organizations also have a role to play in providing informal support and advice to laboratories seeking assistance with validation activities and formal WOAH kit certification processes (Brown *et al.*, 2021).

Validation templates and supporting documentation

Validation templates, which guide users through the process of performing a validation study, can greatly assist in complete and transparent reporting and facilitate objective assessment of validation parameters and fitness-for-purpose(s). The development of validation templates is a useful and practical exercise to guide the planning, implementation, and presentation of validation data. Templates are useful at an institutional level to establish minimum criteria for validating new assays across diagnostic laboratories providing a transparent structure so that gaps in validation data are readily identifiable. Standardising procedures for validation studies and data presentation supports the quality framework required by ISO/IEC17025:2017, and consistent and thorough data presentation not only assists laboratories applying for assay accreditation but simplifies transparency and objective assessment for the auditor.

Validation templates are currently being offered by the WOAH (WOAH, 2022) and other national regulatory bodies such as the Subcommittee on Animal Health Laboratory Standards (SCAHLS) in Australia (DAWE, 2022).

The WOAH template must be used when applying for formal registration of a diagnostic kit and the thoroughness with which assays should be validated can seem a daunting task (WOAH, 2022). However, there are many resources available to help plan and direct validation and verification studies, including the relevant chapters of the WOAH Terrestrial Manual (WOAH, 2018; WOAH, 2019a; WOAH, 2019b; WOAH, 2019c; WOAH, 2019d; WOAH, 2019e; WOAH, 2019f; WOAH, 2019g; WOAH, 2019h) and the recent special issue of the *OIE Scientific and Technical Review on Diagnostic Test Validation* (Saah, 1997; Crowther *et al.*, 2006; Bossuyt *et al.*, 2015; Van Borm *et al.*, 2016; Reising *et al.*, 2021; Watson, Carlile and Williams, 2021; Waugh and Clark, 2021; Johnson and Cabuang, 2021; Gardner *et al.*, 2021; Newberry and Colling, 2021; Gifford *et al.*, 2021; Heuer and Stevenson, 2021; Brown *et al.*, 2021; Halpin *et al.*, 2021; Michel *et al.*, 2021; Kirkland and Newberry, 2021) and guidance documents published by accreditation bodies.

The SCAHLS has approved 17 assays in use in Australian veterinary laboratories using template-based processes (DAWE, 2022). During 2019/2020 the Australian LEADDR network revised two templates (for molecular and serological assays) for the submission of validation data to SCAHLS. The templates closely align with, and indeed are structured around, Stages 1–3 of the WOAH assay validation pathway (DAWE, 2022). Although the primary purpose of the templates was to simplify and standardise validation data for submission to SCAHLS, the templates can also be used by the laboratories as planning tools for validation studies and for collating and presenting validation data for in-house record keeping or external accreditation.

Standards

The current WOAH Assay Development and Validation Pathway (WOAH, 2018) was published in 2013. Additional validation and verification specific chapters have been added to the WOAH Terrestrial Manual over time (WOAH, 2019a; WOAH, 2019b; WOAH, 2019c; WOAH, 2019d; WOAH, 2019e; WOAH, 2019f; WOAH, 2019g; WOAH, 2019h), and additional publications regarding new approaches and methods have communicated gold-standard and increasingly BLCM approaches to specific validation and verification activities. However, wholesale review of the WOAH pathway, to incorporate new knowledge and address the challenges and opportunities highlighted herein, will ensure it continues to meet the needs of WOAH member states in the face of evolving and emerging technologies, methods and diseases.

Specifically, updating standards for reporting of diagnostic accuracy studies may facilitate greater quality and completeness, objectivity and transparency. The human-diagnostic focussed Standards for Reporting of Diagnostic Accuracy (STARD) statement was updated in 2015 and presents a comprehensive checklist for proper design, statistical analysis and reporting of validation and accuracy studies for infectious diseases. The STARD guidelines have been used for specific veterinary purposes including aquatic diseases, paratuberculosis and BLCM (Bossuyt *et al.*, 2015; Heuer and Stevenson, 2021; Kostoulas *et al.*, 2021). However, there exists an opportunity for broader application and integration of this approach into animal health regulatory standards.

Certification schemes

Providing opportunities for recognition, certification and accreditation of an assay may provide commercial incentives for test developers to improve the design, execution and reporting of validation studies.

The WOAH Secretariat for Registration of Diagnostic Kits (WOAH SRDK) provides an internationally recognised certification for diagnostic test kits that can demonstrate fitness-for-purpose (Gifford *et al.*, 2021). Assay developers can apply for registration through submission of validation dossiers, collated with the assistance of an WOAH-developed template. An independent panel of experts assess the validation data provided in the application against the standard of the WOAH Assay Development and Validation Pathway (WOAH, 2018). If successful, the diagnostic kit is added to the WOAH register and comprehensive summaries of the diagnostic performance and fitness-for-purpose is published for potential kit users and regulatory bodies to review (Gifford *et al.*, 2021). Currently, 14 kits have been registered through this scheme (WOAH, 2022).

Other registration schemes may be administered by national regulators. The Subcommittee on Animal Health Laboratory Standards in Australia have approved 17 assays (DAWE, 2022) using a similar template-based process to the WOAH.

5. Conclusions

Adequate validation and verification of the performance characteristics of diagnostic tests for infectious diseases is critical to ensuring that assays are applied and interpreted in a scientifically robust and defensible manner. Since its publication in 2013, the WOAH Assay Development and Validation Pathway (WOAH, 2018) has acted as the internationally recognised standard for the validation of veterinary diagnostic tests for infectious diseases. Whilst the WOAH standard outlines a comprehensive approach to test validation, experience has demonstrated that assay developers continue to face a number of critical challenges in complying with many stages of the pathway. In identifying these obstacles, opportunities for improvement in diagnostic test validation standards, approaches and extension activities have been identified.

International leaders in the field of diagnostic test validation, including the WOAH and their associated Reference Laboratories and Collaborating Centres, as well as national regulatory bodies, have an important role to play in continuing to develop the standards and systems required to ensure that assay developers have the resources required, and incentives, to meet their responsibilities to perform and report well designed and transparent validation studies. End users of diagnostic tests must also be supported to take responsibility to understand and verify assay performance in their own laboratories, and clearly communicate the uncertainty associated with diagnostic test results to their stakeholders.

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Challenges for better livestock production in the developing world

Characterisation of the ovarian cycle in Syrian Awassi ewes using progesterone and oestradiol radioimmunoassay

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Abstract

An experiment was conducted on Syrian Awassi ewes to characterise the oestrous cycle, monitor activity of the corpus luteum and determine normal serum progesterone (P4) and oestradiol (OE2) concentrations during the different phases of the oestrous cycle by using radioimmunoassay. Eighteen cycling Awassi ewes, aged about three years and weighing an average of 57.6 kg, were used during the normal breeding season for two consecutive oestrous cycles. Results showed a cyclic pattern in the P4 and OE2 serum concentration. The average length of 36 oestrous cycles was 17.0 days, divided into: 3.7 days where P4 concentration was low, averaging 1.03 nmol L^{-1}; followed by a sharp increase, which lasted an average of 9.8 days, with a mean P4 concentration of 8.98 nmol L^{-1} (luteal phase); followed by a sharp decline in P4 concentration (follicular phase) that lasted an average of 3.5 days, with a mean P4 concentration of 0.56 nmol L^{-1}. Serum OE2 concentration, despite fluctuations, also showed a cyclic pattern. It was slightly high during the first 3 days of the oestrous cycle (22.91, 26.97, 35.27 pmol L^{-1}), followed by a concentration of around 20 pmol L^{-1} (18.73–21.37 pmol L^{-1}) for a duration of 10 days, and then increased in concentration to reach a maximum of 58.5 pmol L^{-1}, one day before the end of the cycle. A negative and significant correlation ($r = -0.56$, $P < 0.05$) was found between serum P4 and OE2 concentrations.

Keywords

oestrous cycle, progesterone, oestradiol, radioimmunoassay, Awassi ewes

1. Introduction

The oestrous cycle can be defined as a rhythmic change occurring in the reproductive system of females starting from one oestrus phase to another (Pal and Rayees Dar, 2021). Their cycle is regulated by a variety of hormones of hypothalamic, pituitary and ovarian origin. Progesterone (P4) and oestradiol (OE2) are steroid ovarian hormones that have several practical biological functions in the management of reproduction. Therefore, assessment of P4 and/or OE2 concentrations during different physiological stages in animals is a vital exercise, since these two hormones are considered the most important reproductive hormones and have been used widely as a valuable tool to delineate the status of the reproductive performance (Kocakaya *et al.*, 2019; Socheh *et al.*, 2019).

Characterisation of the different phases of the oestrous cycle in animals is considered to be one of the cornerstones of reproductive studies, particularly in seasonal breeds of animals such as the Awassi sheep. The oestrous cycle has been characterised in several animal species including dwarf goats (Khanum, Hussain and Kausar, 2008); crossbred cattle (Mekonnin *et al.*, 2017); miniature

pigs (Noguchi *et al.*, 2015); Arabian mares (Amer *et al.*, 2008); pygmy hippopotamus (Flacke *et al.*, 2017); and red deer (Garcia *et al.*, 2003). In ewes, the oestrous cycle has been hormonally characterised in several breeds such as Olkuska in Poland (Zieba *et al.*, 2002); Bangladeshi sheep (Talukder *et al.*, 2018); Karaman in Türkiye (Arsoy and Sağmanlıgil, 2018); Ghezel in the Islamic Republic of Iran (Najafi *et al.*, 2014); and Swakara and Damara in Namibia (Kandiwa *et al.*, 2019).

Awassi, a fat-tail triple-purpose sheep, is the main native breed in the Syrian Arab Republic (population of more than 14.5 million as compared to 1.8 million for goats and 0.8 million for cows in 2019: MAAR, 2021), and has the widest global distribution of any sheep breed of non-European origin (Galal *et al.*, 2008). Owing to the good quality, and despite the higher prices as compared to other sheep breeds, many consumers prefer the meat and milk of Awassi sheep. In addition, the Awassi wool is suitable for the carpets (Sunderman and Johns, 1994; Tabbaa *et al.*, 2001).

The production of Awassi sheep contributes to the livelihoods of many poor producers and families in the Near Eastern countries, mainly Türkiye, Syrian Arab Republic, Jordan, and Lebanon (Iñiguez *et al.*, 2008). This breed is calm, easily milked, has the advantage of natural hardiness, and is well adapted to grazing production systems (Haile *et al.*, 2017).

The oestrous cycle of the Awassi breed has never been specifically and hormonally studied. Therefore, the main objectives of the current experiment were: 1) to characterise precisely the different phases of the cycle; 2) to determine the normal serum P4 and OE2 concentrations and patterns using radioimmunoassay; and 3) to study the relationship between these two hormones throughout the oestrous cycle of a group of Syrian Awassi ewes.

2. Materials and methods

Location and experimental animals

The experiment was carried out at the Division of Animal Production at Der Al-Hajar area, 33 km south-east of Damascus. This is a dry area and a part of the Syrian steppe region where the majority of the Syrian sheep population is raised. Annual rainfall averages around 120 mm, occurring mainly in December and January.

Eighteen healthy cycling Syrian Awassi ewes with no previous ill-health records, aged about three years and weighing (Mean ± SD) 57.6 ± 4.5 kg, were used in the study, which took place during the breeding season. To determine the duration and hormonal characteristics of the different phases of the oestrous cycle in the Awassi ewes, ovulation was synchronised by using intravaginal sponges containing 40 mg of flugestone acetate (FGA, Intervet, the Netherlands) for 14 days.

Animal housing and feeding

Ewes were kept indoors at night and outside for most of the day. Indoors, the ewes were offered diets based on barley and wheat straw supplemented by vitamins. Outdoors, they had free access to natural grazing. Water and mineral licks were available *ad libitum*.

Blood sampling and hormone analysis

Blood samples (10 mL) were taken daily from the jugular vein of all ewes for 45 days, covering the duration of two consecutive oestrous cycles, starting one day after the removal of the intravaginal sponges. Serum was harvested and stored at -20 °C until assayed. Validated P4 and OE2 radioimmunoassay (RIA) kits (COAT – A – COUNT, DPC, USA) were used. All P4 levels equal to or exceeding 3.18 nmol L^{-1} were considered indicative of normal luteal function, and levels under 3.18 nmol L^{-1} were considered indicative of anoestrous, follicular, or early luteal phase of the oestrous cycle (Zarkawi, 1997).

Statistical analysis

Means of the studied parameters were subjected to an analysis of variance (ANOVA) test by using the Statview-IV program (Abacus Concepts, Berkeley, CA, USA) at the 0.05 probability level. In addition, the coefficients of correlation between P4 and OE2 concentrations were calculated throughout the oestrous cycle based on daily means for each hormone.

3. Results

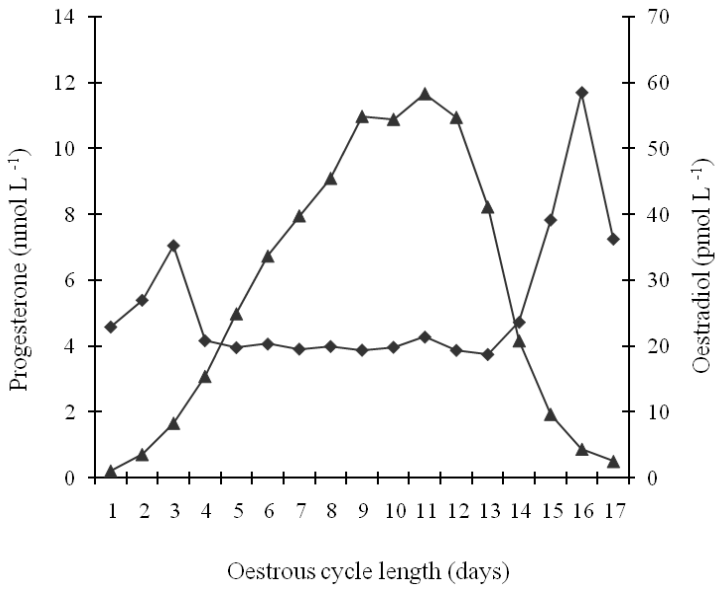
Serum progesterone concentrations

The pattern of the P4 concentration was cyclic: low, followed by a steady increase, then a sharper decrease to a low concentration (Figure 1).

The mean length of the 36 oestrous cycles was found to be 17.0 ± 0.7 days, ranging between 16–18 days. The majority of ewes (58.3 percent) had an oestrous cycle that lasted 17 days, whereas 19.5 percent and 22.2 percent had cycles lasting for 16 and 18 days, respectively. Overall mean serum P4 concentration during the oestrous cycle was 5.61 ± 2.64 nmol L^{-1}.

Hormonally, and based on the activity of the corpus luteum, the oestrous cycle was divided into three phases:

- *Early luteal phase*
  The average length of this phase was found to be 3.7 ± 0.8 days, ranging between 2 and 5 days, with an overall mean P4 concentration of 1.03 ± 0.93 nmol L^{-1}, ranging between 0.01 and 3.14 nmol L^{-1}. The majority of ewes (77.78 percent) had an early luteal phase lasting for two to three days.
- *Luteal phase*
  The length of this phase averaged 9.83 ± 1.0 days (range: 8–12 days), with a mean P4 concentration of



Source: Author's own elaboration.

Figure 1: Patterns and serum concentrations of progesterone (▲) and oesradiol (♦) during the oestrous cycle of Syrian Awassi ewes (n=36)

8.98 ± 3.65 nmol L^{-1} (range: 3.24–26.49 nmol L^{-1}). The maximum mean daily concentration of P4 during this phase was 11.71 ± 3.14 nmol L^{-1}, occurring on day 11 of the oestrous cycle.

- *Follicular phase*
  This phase had an average length of 3.5 ± 0.8 days (range: 2–5 days), with a mean P4 concentration of 0.56 ± 0.60 nmol L^{-1} (range: 0.10–2.77 nmol L^{-1}). Similar to the early luteal phase, the majority of ewes (77.78 percent) had a follicular phase lasting between two and three days.

Thus, P4 concentrations were less than 3.18 nmol L^{-1} for an average of 7.2 ± 0.9 days, with a mean P4 concentration of 0.80 nmol L^{-1}. These days represented the early luteal and follicular phases. The P4 concentrations were greater than 3.18 nmol L^{-1} for an average of 9.83 ± 1.0 days, during which the mean P4 concentration was 8.98 nmol L^{-1}. The period was the luteal phase of the oestrous cycle.

Serum oestradiol concentrations

We also observed a cyclical pattern in serum OE2 concentration during the oestrous cycle (Figure 1). The concentration was found to increase during the first three days (22.91, 26.97, 35.27 pmol L^{-1} for days 1 to 3, respectively), followed by a period of stable concentrations of around 20 pmol L^{-1} (18.73–21.37 pmol L^{-1}) that lasted for 10 days, and a sharp increase thereafter during the days immediately before the end of the oestrous cycle (58.5 pmol L^{-1}), with a sharp decline on the last day. The minimum and maximum serum concentrations of OE2 observed during the oestrous cycle were 7.2 and 150.67 pmol L^{-1}, respectively, whereas the overall mean concentration was 30.67 ± 13.42 pmol L^{-1}.

Relationship between serum concentrations of progesterone and oestradiol

During the oestrous cycle, the mean P4 concentration (5.610 nmol L^{-1}) was more than 180 fold higher than that of OE2 (0.031 nmol L^{-1}). A negative and significant correlation ($r = -0.56$, $P < 0.05$) was found between daily values of serum P4 and OE2 concentrations during the different phases of the oestrous cycle.

4. Discussion

Owing to the importance of sheep in general and Awassi breed in particular, and since there was no information in the literature on the endocrinology of the oestrous cycle of the Awassi ewe, there was a need to perform such a study. Consequently, the results reported herein will be the basis for further reproductive studies aiming at characterisation of some reproductive parameters as a continuation to previous works on Syrian Awassi sheep (e.g. Zarkawi and Soukouti, 2012).

Moreover, determination of the lengths of the different phases of the oestrous cycle is useful in both teaching and research purposes, as well as in assessing the effects of various factors impacting reproductive function (Ajayi and Akhigbe, 2020). Moreover, assessment of P4 and/or OE2 concentrations during different physiological stages in animals is an important activity, since these two hormones are considered the most important reproductive hormones and have been used widely as a valuable tool to delineate the status of the reproductive performance (El-Battawy, 2006; Zarkawi, 2010; Zarkawi and Al-Daker, 2018).

The oestrus cycle length reported here (17.0 days) is similar to the 17.0 days reported for Swakara and Damara sheep in Namibia (Kandiwa *et al.*, 2019), and is close to

the 17.3 days observed among Pelibuey and Suffolk sheep in Mexico (Rodríguez *et al.*, 2009), but higher than the 15.8 days in indigenous ewes in Bangladesh (Zohara *et al.*, 2014). In Brazil, Rodrigues *et al.* (2007) studied the length of the oestrous cycle of 3 breeds (Santa Ines, Romney Marsh and Suffolk) and reported breed differences (17.0, 16.5 and 15.9 days, respectively), but (Kandiwa *et al.*, 2019) did not report differences among 2 sheep breeds (Swakara and Damara) (17.0 days).

The serum P4 concentration pattern was cyclic, coinciding with the different phases of the oestrous cycle: low at the beginning of the oestrous cycle with no active corpora lutea, and with the formation of active corpora lutea thereafter, the secretion of P4 was enhanced during the luteal phase. Since no pregnancy occurred, the concentration dropped sharply as a result of the luteolysis of the corpora lutea. In the current study, overall mean serum P4 concentration during the oestrous cycle was 5.61 ± 2.64 nmol L^{-1} ranging from 0.01 to 26.49 nmol L^{-1} based on the different phases. The pattern of P4 concentration during the different phases of the oestrous cycle (low, high and low) is similar to that reported for Ghezel sheep (Najafi *et al.*, 2014).

Differences in the minimum and maximum values in P4 and OE2 concentrations were found among animals. This could be attributed to individual variations within the breed. Similar differences in P4 concentrations in sheep breeds were reported in Finnsheep, Friesian, Polish Heath, Zelanza and Karakul in Poland (Reklewska *et al.*, 1988) and in Menz in Ethiopia (Mukasa-Mugerwa *et al.*, 1990).

In white Karaman ewes, Arsoy and Sağmanlıgil (2018) reported that after Day 4, there was an accelerated rise in P4, with maximum concentration (3.73 nmol L^{-1}) reached on Day 11. Between Days 11and 15, P4 concentrations remained high, then fell suddenly to a basal level after day 15. The overall concentration during the oestrous cycle ranged from 0.01 to 9.0 nmol L^{-1}. In indigenous ewes in Bangladesh, Zohara *et al.* (2014) indicated that on Days 0 to 5 of the cycle the P4 concentration was 0.29 to 5.09 nmol L^{-1}, and on 5–10 days ranged from 5.09 to 8.9 nmol L^{-1} with maximum concentration of 8.9 nmol L^{-1} observed on Day 10 of the oestrous cycle. Thereafter it dropped rapidly to a basal level of 0.35 nmol L^{-1} on Day 0. The authors indicated that the pattern of P4 concentration could be used to define the reproductive stages of indigenous ewes. El-Zaher *et al.* (2020) studied the effects of thyme, celery and salinomycin on ovarian sex hormones during the oestrous cycle of Bakri ewes. They reported that thyme and celery and the mixture of the two increased ($P < 0.01$) OE2 during the follicular phase of the oestrous cycle, relative to the untreated control animals. In contrast, only animals fed celery showed a marked ($P < 0.001$) increase in P4 during the luteal phase. They concluded that the treatments had significant effects on reproductive performance in ewes throughout the oestrous cycle.

The serum OE2 concentration pattern was also cyclic in this study, but with a generally opposite shape to that found for the P4. This was reflected by the negative and significant correlation between the daily concentrations of the two hormones during the oestrous cycle. Serum P4 and OE2 patterns in the Syrian Awassi ewes followed the luteal and follicular phases of the oestrous cycle; however, the P4 pattern was more reliable in assessing the different phases of the oestrous cycle. The P4 and OE2 patterns found in this study were similar to that reported by Herriman *et al.* (1979) in study of Dorset Horn x Finnish Landrace sheep in the United Kingdom.

Arsoy and Sağmanlıgil (2018) reported mean plasma OE2 concentration of 31.67 pmol L^{-1} during the oestrous cycle in Karaman ewes, which decreased towards Day 8 of the cycle and then increased again on Day 11: 22.36 pmol L^{-1}. It decreased to its minimum concentration at the end of the cycle (Day 15; 12.85 pmol L^{-1}).

Despite the cyclic pattern, the mean daily OE2 concentrations showed minor fluctuations until Day 14 and then gradually increased from 23.62 to reach a peak of 58.5 pmol L^{-1} one day before the end of the oestrous cycle. Zieba *et al.* (2002) reported similar results in the serum concentrations of OE2 in Olkuska ewes reaching 20.08 and 48.25 pmol L^{-1} on Days 14 and on the day before ovulation during the oestrous cycle, respectively. The latter authors concluded that the preovulatory follicles originated from the large follicle population that were present in the ovary at the time of luteal regression. This also confirms the finding of Gonzalez-Bulnes *et al.* (2001) stating that in Mouflon sheep in Spain, the single ovulatory follicle is the largest growing follicle present in the two ovaries at the time of luteolysis. It will be selected to grow and ovulate, while development of other follicles will be inhibited.

5. Conclusions

Four main results were observed in this study:

- We characterised for the first time the oestrous cycle of the local Syrian Awassi ewes and determined precisely the normal serum P4 and OE2 patterns and concentrations during different phases of the oestrous cycle.
- Serum P4 and OE2 concentrations showed a cyclic pattern.
- A negative and significant correlation between the daily concentrations of the two hormones was established during the oestrous cycle.
- The P4 concentration (5.60 nmol L^{-1}) was more than 180 times larger than that of OE2 (0.031 nmol L^{-1}), indicating that only minute concentrations of the latter were necessary to have an effect on the growth and development of the ovarian follicles.

Assessing the physiology of the oestrous cycle of the Syrian Awassi ewes will be useful for future reproductive, genetic and/or breeding studies, pertaining to or in dealing with this particular breed of sheep.

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Dairy goat production in India: A review

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Abstract

The global dairy goat industry is expanding rapidly, and this growth is expected to continue in the future. This expansion follows trends seen for livestock in general, as demand for animal source products are increasing globally in view of sizable increases in per capita income and health consciousness of people. Dairy goat farming is slowly gaining momentum in India and niche markets seem to be increasing for using goat milk in ayurvedic medicinal preparations, cheese production and for lactose intolerant people. The goat population of India was 148.88 million in 2019, which represents an increase of 10.14 percent over the previous census period (2012) and is 27.8 percent of the total national livestock population. About 95 percent of the goats in India are reared in rural areas and contribute greatly to the rural economy and help meet the sustenance of the rural poor people. The top ten goat rearing states in India are Rajasthan (14.0 percent), West Bengal (10.9 percent), Uttar Pradesh (9.7 percent), Bihar (8.6 percent), Madhya Pradesh (7.4 percent), Maharashtra (7.1 percent), Tamil Nadu (6.6 percent), Jharkhand (6.1 percent), Odisha (4.3 percent) and Karnataka (4.1 percent). The milk production from goats increased from 0.535 million tonnes in 1961 to 5.40 million tonnes in 2019. The top five states in terms of goat milk production (estimated) in India are Rajasthan, Uttar Pradesh, Madhya Pradesh, Gujarat and Maharashtra. Based on the existing information, goat milk is marketed only to a small extent and largely through informal channels. Recent initiatives on commercial goat farming and processing have focused on goat milk to capitalize on the nutritional value and market for high value processed products. India is comparatively rich in genetic resources, when compared to other countries and these resources, together with the on-going genetic improvement programmes supported by the Government of India for dairy and dual-purpose breeds, provide a path to successful expansion of the dairy goat industry in India.

Keywords

goat, breeds, milk production, India

1. Introduction

The global dairy goat industry is expanding rapidly, and the overall goat industry has the potential to provide sustainable livelihoods, especially in limited resource areas, and enable smallholders to accumulate assets. In addition to milk, dairy goats provide other benefits to owners, including meat, skins for leather, kids to be sold for breeding or slaughter, and manure for fertilizer (Miller and Lu, 2019). Asia is often called the home of the goat because the region hosts 60 percent of the world goat population. They are often raised under harsh environmental conditions, but they are among the major providers of nutrition, food security and socioeconomic status to their human owners. In addition to wholesome and nutritious milk-based products, dairy goats provide sustainable livelihoods, especially in limited resource areas, and enable smallholders to accumulate assets (Lu, 2017b; Miller and Lu, 2019; Liang and Paengkoum, 2019).

In India, development of the goat sector has the potential to impact the livelihoods of 20 million goat rearers (NSSO, 2013). Most (83.4 percent) goat rearers are landless, smallholder and marginal farmers, and goat rearing dominates in ecologically vulnerable and drought prone areas (NAP, 2017; Report, 2018a; Thiruvenkadan and Chidananda, 2018; Report, 2019a; Report, 2019b). Among all species of farm animals, goats have the widest ecological range and have been poor people's most reliable livelihood resource since their domestication during Neolithic Revolution about 10 millennia ago. Goat rearing is a traditional backyard activity supplementing household income, and contributes about 10 to 40 percent of household income in different regions. Goat husbandry in India is essentially an endeavour of millions of small holders who rear animals on crop residues and common

property resources. Dairy goat farming is slowly gaining momentum in India as indicated by the increase in milk production over the decades i.e. from 0.535 million tonnes in the year 1961 to 5.4 million tonnes in the year 2019 (Report, 2019b), and the demand for goat milk and milk products for internal consumption and export is expected to rise in coming years (NAP, 2017; Report, 2019b).

2. Growth pattern of different livestock in India and population statistics of goats

Table 1 shows annual livestock population numbers in India by species since 1951. The goat population of India is 148.88 million (2nd highest after China) and has been increasing by 0.5 percent annually during 2007 to 2019 (Report, 2019b) with about 56 percent slaughter and 15 percent mortality. The Indian goat population has been rising marginally since the 1960s, due to changing incomes and food preferences in human populations, and climate change limiting areas for raising cattle, buffalo and sheep. The growth rates of goat, sheep and buffalo and cattle populations from 1951 to 2019 have been 144.50, 84.82, 114.65 and 9.62 percent respectively. About 95 percent of the goats in India are reared in rural areas and contribute greatly to the rural economy to meet the sustenance of the rural poor people (NAP, 2017; Thiruvenkadan and Chidananda, 2018; Report, 2019b).

The percentage changes of different livestock populations of India over the decades is presented in Table 2. The table indicates a positive growth rate in cattle, buffalo, sheep and goat in most of the periods. The goat population of India observed an increase of 10.14 percent (Figure 1) over the previous census period and the total goat population as of 2019 is 27.8 percent of the total livestock population of the country.

The top ten goat rearing states in India are Rajasthan (14.0 percent), West Bengal (10.9 percent), Uttar Pradesh (9.7 percent), Bihar (8.6 percent), Madhya Pradesh (7.4 percent), Maharashtra (7.1 percent), Tamil Nadu (6.6 percent), Jharkhand (6.1 percent), Odisha (4.3 percent) and Karnataka (4.1 percent). There is a slight fluctuation in goat populations between different states (Figure 2) between different census periods (Report, 2012; Report, 2017; Report, 2019b).

3. Goat milk production statistics

Global goat milk production statistics are presented in Table 3. The most important goat milk producing countries are in Asia, Europe, Near East and Africa. Among the Asian nations, South Asian countries viz., India, Bangladesh and Pakistan are the major producers. The total global goat milk production as of 2019 was estimated at 19.91 million tonnes with an increase of about 95.9 percent from 1990 to 2019. During this period, Asia has seen the largest increase in goat milk production (112.95 percent), followed by Africa (112.33 percent), America (71.27 percent), Europe (42.26 percent) and Oceania (56.00 percent) (FAOSTAT, 2019).

Goat milk production has increased gradually since the 1990s especially in Asia (Figure 3) and the increased production might be due to increased demand for dairy goat products in traditional markets, as well as development of new markets. The milk production from goats in the Asiatic region increased from 2 417.26 thousand tonnes in the year 1961 to 11 682.19 thousand tonnes in the year 2019, whereas the Africa region witnessed steady growth from 1 309.06 thousand tonnes to 4 352.72 thousand

Table 1: Livestock population of India (in millions) from 1951 to 2019

Species	1951	1956	1961	1966	1972	1977	1982	1987	1992	1997	2003	2007	2012	2019
Cattle	155.3	158.7	175.6	176.2	178.3	180.0	192.5	199.7	204.6	198.9	185.2	199.1	190.9	192.5
Buffalo	43.4	44.9	51.2	53.0	57.4	62.0	69.8	76.0	84.2	89.9	97.9	105.3	108.7	109.9
Total bovines	**198.7**	**203.6**	**226.8**	**229.2**	**235.7**	**242.0**	**262.2**	**275.7**	**288.8**	**288.8**	**283.1**	**304.4**	**299.6**	**302.3**
Sheep	39.1	39.3	40.2	42.4	40.0	41.0	48.8	45.7	50.8	57.5	61.5	71.6	65.1	74.3
Goat	47.2	55.4	60.9	64.6	67.5	75.6	95.3	110.2	115.3	122.7	124.4	140.5	135.2	148.9
Total livestock	**292.9**	**306.6**	**336.5**	**344.5**	**353.2**	**369.4**	**419.6**	**445.2**	**470.9**	**485.4**	**485.0**	**529.7**	**512.1**	**535.8**
Percent goat/Total livestock	**16.11**	**18.07**	**18.10**	**18.75**	**19.11**	**20.47**	**22.71**	**24.75**	**24.49**	**25.28**	**25.65**	**26.52**	**26.40**	**27.79**

Sources: a Report. 2012. 19th Livestock Census. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi. www.sapplpp.org/sheep-goat-poultry-statistics/common-resources/19th-livestock-census-of-india-an-overview.html#.YmwvNy8r3IE

b Report. 2017. Basic Animal Husbandry Statitics-2017 AHS series 18. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi. www.scribd.com/document/392701346/Basic-Animal-Husbandry-and-Fisheries-Statistics-2017-English-Version-5

c Report. 2019b. Key results of Animal Husbandry Statitics-2019. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi.

tonnes. The milk production in the European region has oscillated between 2 890.57 and 3 075.26 thousand tonnes over the decades. In Asia, India, Bangladesh and Pakistan alone produced 9 090 thousand tonnes of goat milk (as of 2019) and accounted for 77.81 percent of the total goat milk produced in that region and also accounted for 45.66 percent of the total world goat milk production (FAOSTAT, 2019). In general, the goat milk and milk products are increasingly preferred for their health and nutritional benefits, including greater digestibility and enhanced lipid metabolism, in addition to their taste, compared to cow milk. The attractive price for goat products, especially milk, has brought new producers and investors into the field. The potential for the goat milk industry is quite promising especially for low- and medium-income people (Haenlein, 2004; Park, 2010; Liang and Paengkoum, 2019).

Table 2: Growth pattern of the livestock population of India from 1951 to 2019

Species	Annual growth rate (%)												
	1951–56	1956–61	1961–66	1966–72	1972–77	1977–82	1982–87	1987–92	1992–97	1997–2003	2003–07	2007–12	2012–19
Cattle	2.19	10.65	0.34	1.19	0.95	6.92	3.76	2.45	-2.79	-6.89	7.50	-4.10	0.8
Buffalo	3.46	14.03	3.52	8.30	8.01	12.55	8.87	10.85	6.78	8.90	7.58	3.19	1.0
Sheep	0.51	2.29	5.47	-5.66	2.50	18.93	-6.28	11.12	13.21	6.92	16.41	-9.07	14.1
Goat	17.37	9.93	6.08	4.49	12.00	25.99	15.71	4.60	6.45	1.34	13.01	-3.82	10.1

Sources: a Report. 2012. 19th Livestock Census. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi. www.sapplpp.org/sheep-goat-poultry-statistics/common-resources/19th-livestock-census-of-india-an-overview.html#.YmwvNy8r3IE
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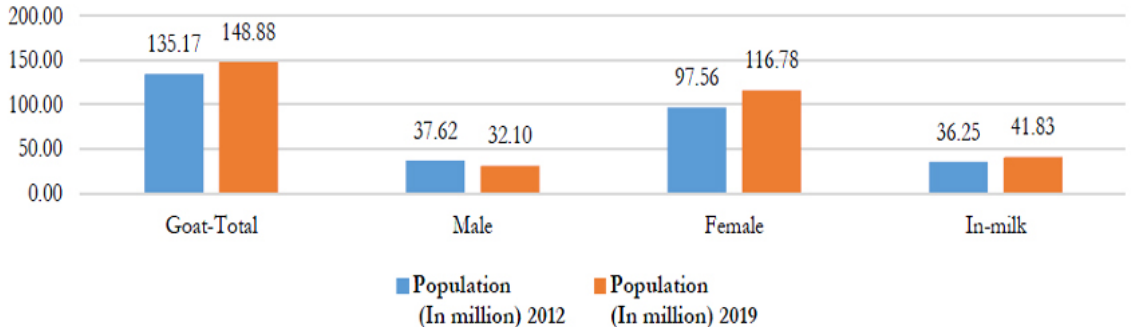
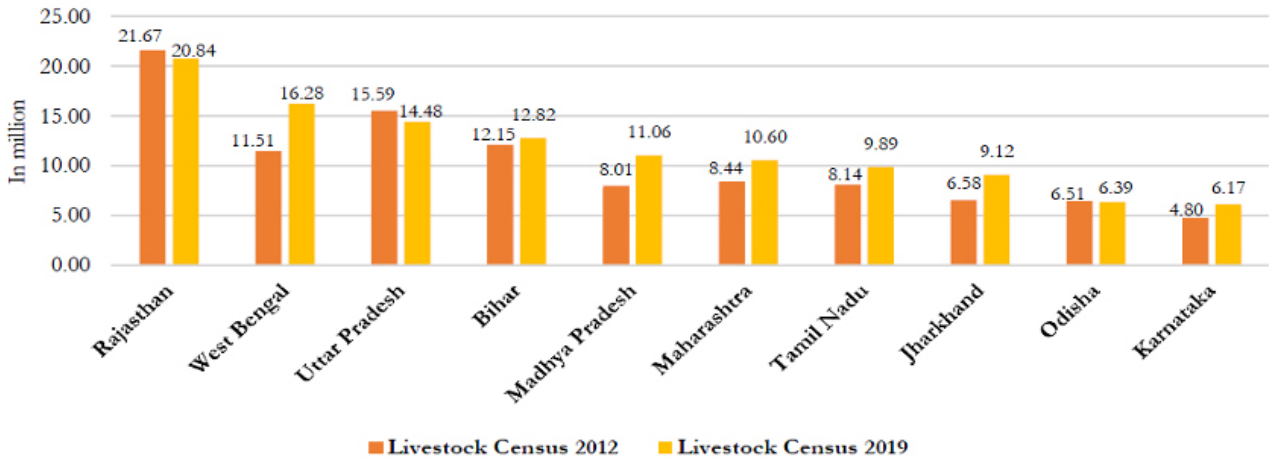


Source: Report. 2019b. Key results of Animal Husbandry Statitics-2019. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi.

Figure 1: Comparison of goat populations between 2012 and 2019 in India



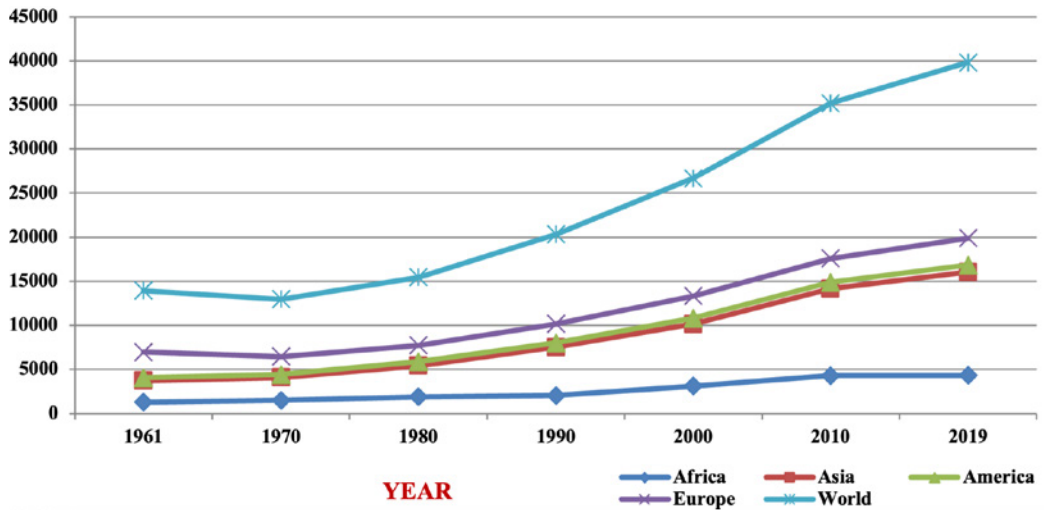
Source: Report. 2019b. Key results of Animal Husbandry Statitics-2019. Ministry of Agriculture, Dairying and Fisheries, Government of India, New Delhi.

Figure 2: Goat populations in 2012 and 2019 for the major states of India

Table 3: Goat milk production (1 000 tonnes) from 1961 to 2019

Countries	1961	1970	1980	1990	2000	2010	2019
India	535.00	624.00	947.00	2 381.00	3 266.00	4 594.00	5 400.00
Bangladesh	291.84	347.76	353.60	807.60	1 312.00	2 496.00	2 750.00
Sudan	309.00	296.00	470.00	527.69	1 245.00	1 512.00	1 158.00
Pakistan	86.20	176.00	406.00	500.00	586.00	739.00	940.00
France	250.00	279.00	453.00	430.83	473.84	645.18	656.74
Spain	332.10	314.50	298.89	487.66	438.54	522.11	535.79
Somalia	362.00	440.00	543.34	540.00	390.00	410.00	373.40
Indonesia	124.00	104.00	120.00	180.00	268.89	335.16	369.58
Greece	258.29	347.37	414.38	498.61	520.27	471.68	355.76
Iran (Islamic Republic of)	366.50	409.60	631.80	643.40	357.98	197.79	272.08
China	45.00	54.00	107.27	165.70	230.01	277.23	236.44
Italy	204.89	143.18	118.40	128.60	112.80	24.94	62.34
Total	**3 164.82**	**3 535.41**	**4 863.68**	**7 291.10**	**9 201.32**	**12 225.09**	**13 110.13**
			Continent				
Africa	1 309.06	1 522.84	1 900.20	2 050.05	3 119.53	4 306.29	4 352.72
Asia	2 417.26	2 571.65	3 519.93	5 485.69	7 022.99	9 838.80	11 682.19
America	352.82	328.51	456.61	467.19	678.65	744.46	800.16
Europe	2 890.57	2 059.10	1 856.77	2 161.68	2 515.48	2 704.11	3 075.26
Oceania	0.02900	0.01100	0.01800	0.02500	0.02800	0.04200	0.03900
World	**6 969.74**	**6 482.11**	**7 733.53**	**10 164.64**	**13 336.67**	**17 593.70**	**19 910.38**

Source: FAOSTAT. 2019. *Faostat.org* [online]. Cited 29 April 2022. http://ww1.faostat.org



Source: Author's own elaboration.

Figure 3: Regional goat milk production over recent decades

The percent contribution of goat milk production over the decades by important goat milk producing countries is presented in Table 4. The percent contribution of goat milk from Asia, Africa, Europe and America continent was 58.67, 21.86, 15.45 and 4.02 percent respectively, and India is the highest producer of the goat milk with a share of 27.12 percent followed by Bangladesh (13.81 percent), Sudan (5.82 percent) and Pakistan (4.72 percent). In many countries, official statistics do not include home consumption or informal market sales where records are not kept, complicating efforts to measure the value of goats. Goats in developing countries are frequently kept by small-scale producers, and are not part of specialized production systems, so it can be hard to estimate the contribution of goat milk to livelihoods. Majority of world's goat milk is produced and consumed in India, Bangladesh and Pakistan and goat milk for food and income is also important in the countries of the Mediterranean, the Near East, Eastern Europe and parts of South America. (Lu, 2017a; Lu, 2017b; Miller and Lu, 2019; Liang and Paengkoum, 2019; Report, 2019b).

Table 4: Goat milk production (percent) from 1961 to 2019

Countries	1961	1970	1980	1990	2000	2010	2019
India	7.68	9.63	12.25	23.42	24.49	26.11	27.12
Bangladesh	4.19	5.36	4.57	7.95	9.84	14.19	13.81
Sudan	4.43	4.57	6.08	5.19	9.34	8.59	5.82
Pakistan	1.24	2.72	5.25	4.92	4.39	4.20	4.72
France	3.59	4.30	5.86	4.24	3.55	3.67	3.30
Spain	4.76	4.85	3.86	4.80	3.29	2.97	2.69
Somalia	5.19	6.79	7.03	5.31	2.92	2.33	1.88
Indonesia	1.78	1.60	1.55	1.77	2.02	1.90	1.86
Greece	3.71	5.36	5.36	4.91	3.90	2.68	1.79
Iran (Islamic Republic of)	5.26	6.32	8.17	6.33	2.68	1.12	1.37
China	0.65	0.83	1.39	1.63	1.72	1.58	1.19
Italy	2.94	2.21	1.53	1.27	0.85	0.14	0.31
Total	**45.44**	**54.57**	**62.92**	**71.75**	**69.01**	**69.50**	**65.86**
			Continent				
Africa	18.78	23.49	24.57	20.17	23.39	24.48	21.86
Asia	34.68	39.67	45.52	53.97	52.66	55.92	58.67
America	5.06	5.07	5.90	4.60	5.09	4.23	4.02
Europe	41.47	31.77	24.01	21.27	18.86	15.37	15.45
Oceania	0.00042	0.00017	0.00023	0.00025	0.00021	0.00024	0.00020
World	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

Source: FAOSTAT. 2019. *Faostat.org* [online]. Cited 29 April 2022. http://ww1.faostat.org

Milk from cattle and buffaloes and their products have been synonymous with the concept of the dairy industry for a long time. However, other species also contribute significant amount of milk, such as goat, sheep and camels; and their contributions cannot be overlooked. The milk production of India obtained from cows, buffalos and goats is presented in Table 5. The milk production from dairy animals has increased from 20.57 million tonnes in 1961 to 187.63 million tonnes in 2019, and the total milk production from cows, buffalo and goat in the year 1961 was 8.753, 11.087 and 0.535 million tonnes respectively and the corresponding values in the year 2019 are 90.00, 92.00 and 5.40 million tonnes. The top five states in terms of estimated goat milk production in India are: Rajasthan, Uttar Pradesh, Madhya Pradesh, Gujarat and Maharashtra (FAOSTAT, 2019).

Table 5: Milk production of India from different species over the decades

Year	Cow (1 000 tonne)	Buffalo (1 000 tonne)	Goat (1 000 tonne)	Camel (1 000 tonne)	Sheep (1 000 tonne)	Total (1 000 tonne)	Cow (%)	Buffalo (%)	Goat (%)
1961	8 753	11 087	535	17.013	173.182	**20 565**	42.6	53.9	2.6
1971	9 450	12 375	675	-	-	**22 500**	42.0	55.0	3.0
1981	14 406	18 865	1 029	-	-	**34 300**	42.0	55.0	3.0
1991	23 038	28 675	2 348	-	-	**54 061**	42.6	53.0	4.3
2001	34 516	45 402	3 501	13.485	196.267	**83 629**	41.3	54.3	4.2
2011	57 770	65 352	4 782	9.845	210.172	**128 124**	45.1	51.0	3.7
2019	90 000	92 000	5 400	6.944	226.104	**187 633**	48.0	49.0	2.9

Source: FAOSTAT. 2019. *Faostat.org* [online]. Cited 29 April 2022. http://ww1.faostat.org

4. Production systems of dairy goat in India

In India, goats are reared by men and women with diverse working and professional backgrounds. The production systems are as numerous as the socioeconomic and varied agricultural situations in the country. However, they can be broadly classified into the following:

- **Tethering:** This is common in the sub-humid and humid zones, where probably because of intensive cropping, it is a convenient means of rearing goats from the standpoint of control, minimum labour input and

utilization of feed *in situ*. It is thus a sedentary system. A variation of this method is combining tethering with grazing up to 5 goats at a time, led by ropes held by women and children.

- **Extensive production:** This involves low carrying capacity in situations where land is marginal and is plentiful. It is characterized by low rainfall and various browse plants. The system is used by nomadic people, usually in very low rainfall areas or during winter months when crop residues are available.
- **Intensive production:** The goats are fed in confinement with limited access to land. It involves high labour and cash inputs. Cultivated grasses and agro-industrial by-products are fed *in situ*. This system has the advantage of allowing substantial control over the animals.
- **Semi-intensive production:** This system is practiced to some degree in most of the situations, but the nature and extent of integration depend on the type of crops grown and their suitability to goats. The advantages of this system are increased fertility of land via the return of dung and urine, control of waste herbage growth, reduced fertilizer usage, easier crop management, increased crop yields, and greater economic returns (Thiruvenkadan, 2012; Thiruvenkadan and Rajendran, 2014; NAP, 2017; Thiruvenkadan, 2019).

5. Dairy goat breeds and their production

The European goat sector is highly specialized for goat milk production, mostly for industrial cheese making, although it also supports traditional on-farm manufacturing. The European dairy goat sector is well-regulated, and nearly all goat milk is processed, mostly into cheese and relatively little is consumed directly by the producer's family. Europe owns only 2.5 percent of the world's goat herd, but produces 18 percent of the world's goat milk. It is the only continent where goat milk has considerable economic importance and organization (Dubeuf, 2005; Dubeuf and Boyazoglu, 2009; Dubeuf, de A. Ruiz Morales and Castel Genis, 2010). The main cheeses produced are in European countries (i.e. in France: Bastelicaccia, Sartinese, Venachese, Calinzanincu, Niulincu; in Greece: Feta and other white cheeses; in the Canary Islands: Palmero and Majorero; in Murcia: Murcia cheese; in Portugal: Serra da Estrela, Castelo Branco, Nisa, Évora, Azeitão, Serpa, and Rabaçal) (Dubeuf, de A. Ruiz Morales and Castel Genis, 2010). In some Eastern European countries, a viscous and refreshing drink called "Kefir" is produced from goat milk, and alcohol and carbon dioxide may be added to it (Boutonnet *et al.*, 2016).

Switzerland led in the development of the world's highest milk yielding dairy goat breeds. The important one is the Saanen, and it is widely recognized as the world's best developed and highest milk-producing breed. It has been compared among goat breeds to the Holstein Friesian dairy cattle breed, because of their similarly high levels of daily milk yield and relatively low levels of milk fat content. The name Saanen is derived from the breed origin in the Saanen valley of central Switzerland (Park, 2010; Devendra and Haenlein, 2011). The milk yield of European goat breeds ranges from 1 500–3 000 litre/lactation in a 220–280 day lactation period. Among specific breeds, maximum milk by individual Alpine, Nubian, Saanen and Toggenburg goats has been recorded as 2 916, 2 700, 2 064 and 3 620 kg in a 305 day lactation period, respectively (Thiruvenkadan and Rajendran, 2014; Miller and Lu, 2019). High productivity is the result of the availability of excellent genetics and widespread use of artificial insemination, coupled with high planes of nutrition and good management. Israel is often grouped with the European countries for statistical purposes because of similar levels of technology and production, as well as trade agreements and sanitary regulations. The average production per doe in Israel is 305 kg/year, and is higher on many specialized goat dairy farms. Worldwide, the average production is only 90 kg/year per doe (Van Dijk, 1996; Lu, 2017b; Miller and Lu, 2019; Lu and Miller, 2019).

India has a rich repository of goat genetic resources, and its genetic resources are reflected by the availability of 34 breeds of goats (NBAGR, 2019). These goat breeds are good performers in their habitat under adequate feeding and bear a great degree of resistance and tolerance for disease and climatic fluctuations. The Beetal, Jakhrana, Surti, Gohilwadi and Zalawadi breeds yield 150–300 litres of milk in 150–200 days of lactation and are large in body size and late maturing and originated from the north/northwestern regions of India (Acharya, 1982; Thiruvenkadan, 2012). Generally, the milk productivity of individual goats in India is about 0.46 kg/day per goat (Thiruvenkadan, 2012; Report, 2018a; Report, 2018b; Report, 2019a). These dairy goats have good prolificacy and possess a large variation for lactation and growth performance traits (Acharya, 1982; Thiruvenkadan, 2012; Thiruvenkadan and Rajendran, 2014) and thus may be used as candidate breeds for grading up of the large number non-descript goats in arid and semi-arid regions of India. The direct comparison of Indian goat breeds for milk production is not straight-forward, however, as goats are raised (both between and within breeds) under highly variable feeding and management conditions in different agroclimatic regions (Singh and Chauhan, 2021). The lactation performance of the goat breeds of India can be roughly grouped as in Table 6.

The average body weights and milk yields of the important dairy and dual purpose goat breeds are presented in Table 7.

Table 6: General characteristics of the main dairy and dual purpose goat breeds of India

Type	Dairybreeds	Dual purpose breeds
Purpose	Mostly milk, but also meat	Milk and meat
Breeds	Beetal, Surti, Jhakrana and Malabari	Jamunapari, Sirohi, Barbari, Marwari, Kutchi, Mehsana, Zalawadi and Osmanabadi
Average milk yield	150–300 litre	75–125 litre
Average lactation length	150–200 days	90–150 days

Sources: a Acharya, R.M. 1982. *Sheep and goat breeds of India*. FAO animal production and health paper 30. Rome, Food and Agriculture Organization of the United Nations. 190 pp.
b Thiruvenkadan, A.K. 2012. Improving rural livelihood through dairy goat farming in India. Paper presented at First Asia Dairy Goat Conference, 9–12 April 2012, Corus Hotel, Kuala Lumpur, Malaysia, 2012. www.fao.org/3/i2891e/i2891e00.pdf
c DAHD (Department of Animal Husbandry and Dairying). 2013. https://dahd.nic.in
d Thiruvenkadan, A.K. & Rajendran, R. 2014. Breeding strategies for dairy goats in India. Paper presented at Second Asian-Australasian Dairy Goat Conference, 25-27 April, Bogor, Indonesia, 2014.
e NBAGR (National Bureau of Animal Genetic Resources). 2019. https://nbagr.icar.gov.in/en/home
f NAP. 2017. National Action Plan- Goat- 2022. https://dahd.nic.in/sites/default/filess/NAP%20on%20Goat.pdf
g FAOSTAT. 2019. *Faostat.org* [online]. Cited 29 April 2022. http://ww1.faostat.org

Table 7: Average body weights and daily milk yields of the main dairy and dual purpose goat breeds of India

Sl. No.	Name of breed	Original state	Body weight		Average daily milk yield (kg)
			Buck (kg)	Doe (kg)	
1.	Sirohi	Rajasthan	50.37	22.54	0.41
2.	Marwari	Rajasthan	33.18	25.85	0.53
3.	Beetal	Punjab	59.07	34.97	1.16
4.	Jhakrana	Rajasthan	57.80	44.48	3.18
5.	Jamnapari	Uttar Pradesh	44.66	38.03	1.06
6.	Barbari	Uttar Pradesh	36.70	20.30	0.71
7.	Mehsana	Gujarat	37.00	32.00	1.32
8.	Zalawadi	Gujarat	38.84	32.99	2.02
9.	Kutchi	Gujarat	46.96	39.91	1.84
10.	Surti	Gujarat	29.50	32.03	2.50

Sources: a Acharya, R.M. 1982. *Sheep and goat breeds of India*. FAO animal production and health paper 30. Rome, Food and Agriculture Organization of the United Nations. 190 pp.
b Thiruvenkadan, A.K. 2012. Improving rural livelihood through dairy goat farming in India. Paper presented at First Asia Dairy Goat Conference, 9–12 April 2012, Corus Hotel, Kuala Lumpur, Malaysia, 2012. www.fao.org/3/i2891e/i2891e00.pdf
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g FAOSTAT. 2019. *Faostat.org* [online]. Cited 29 April 2022. http://ww1.faostat.org

6. Consumer preferences for goat milk in India

The preferred dairy animals in India are buffaloes, unlike the majority of the world market, which is dominated by cow's milk. As much as 98 percent of milk in India is produced in rural areas and it caters to 72 percent of the total population. Goat milk is an important supplement for dairy production, particularly for residents in underdeveloped rural areas and hilly mountainous regions. In only a few states (viz. Karnataka, Delhi, Rajasthan, Madhya Pradesh, Punjab and Uttar Pradesh) do consumers prefer goat milk to cows and buffalo milk (Report, 2018b). Goat's milk has specialized markets in these states. To improve the goat milk consumption, small goat milk producers need to capitalize on niche markets by catering to the specific demands for products such as fresh, organic and/or low-fat milk, as well as goat cheese as in European countries. These production systems need to be standardized and supported for better adoption, increased production and better income generation. Benefits of Indian consumers' preference for fresh milk could then directly flow to small milk producers. Policy targeted at strengthening of fresh milk marketing with added attributes viz., fortification will help the small producers. For commercial exploitation of the value-added goat foods, development of shelf stable products assumes greater importance, and work has to be carried out in this direction. Novel functional products such as antioxidant and/or omega-3 fatty acids enriched, low-cholesterol goat milk products, herbal products, low-calorie indigenous goat foods and mineral and vitamin fortified functional products foods might be evaluated. This would require policy intervention on supply chain management of fresh

milk, including its retailing, and direct communication between the end users and producers. In general, most of the goat milk produced is directed to self-consumption, however, marketing of fresh-liquid goat milk is slowly increasing in urban areas (Thiruvenkadan and Chidananda, 2018). Goat's milk is not new in India, but the growth of milk federations for the procurement of cows and buffalo milk saw its consumption diminish as a result of easy access to the cows and buffalo milk. The cost of goat milk is relatively high in part because it is still a very small market in which demand exceeds supply. The price of cow/buffalo milk per kg ranges between Rs. 30/- and 50/- (USD 0.5 to 0.75) at different localities, whereas the price of goat milk per kg ranges between Rs. 100/- and 200/- (USD 1.5 to 2.5) and is not fixed according to the fat content (Thiruvenkadan, 2012; Thiruvenkadan and Rajendran, 2014; Thiruvenkadan and Rajendran, 2015; Thiruvenkadan and Rajendran, 2016).

7. Genetic improvement programmes

In general, in the recent past, the focus of goat improvement programmes in India and market remains on meat production. Therefore, less effort has been made by farmer and development agencies to improve milk productivity and the development of markets for milk. This has resulted in a gradual decline in milk productivity as well as the population sizes of dairy goat breeds. The recent increase in milk demand has, however, again made dairy goat farming economically viable in some instances (Singh and Chauhan, 2021). In order to improve the production potential of goats, the All India Coordinated Research Project (AICRP) has been redesigned with modified objectives and technical programmes to accommodate farmer's herds for long term improvement under their prevailing ecosystems (Report, 2018a; Report, 2019a). This project presently covers 14 goat breeds through 18 centres located across the country, which are coordinated by a dedicated unit located at the Central Institute for Research on Goats, in Makhdoom, Mathura, Uttar Pradesh. Under this scheme, the major dairy and dual-purpose breeds are being considered for improvement of milk as well as meat production. They include the Jamunapari, Barbari, Sirohi, Sangamneri, Surti, Malabari and Osmanabadi goats. The major thrust of the project is to build up long-term capacity of goat keepers through technology demonstration, capacity building and introduction of genetically superior breeding stock for enhancing production and reproduction potential (Thiruvenkadan and Chidananda, 2018).

8. Challenges of dairy goat farming in India

The major constraints in dairy goat farming in India (Thiruvenkadan, 2012; DAHD, 2013; Thiruvenkadan and Rajendran, 2014; DAHD, 2017; FAOSTAT, 2019; NBAGR, 2019) are as follows:

- Non-availability of high-yielding breeding stock and low levels of nutrition and managerial efficiency.
- Lack of definition of the production objectives and limited attention to application of modern techniques for improving the reproductive efficiency of goats.
- Inadequate control of diseases and parasites due to non-availability of prophylactic vaccines against important contagious diseases and well as limited organized marketing support facilities (Thiruvenkadan, 2012).
- In most parts of India, goats are reared mainly for meat production and milk is considered as a secondary source of income or as a by-product. The lack of diversified goat milk products is also one of the common denominators in several regions of India (Thiruvenkadan and Chidananda, 2018).
- Most of the purebred dairy goat populations are declining due to indiscriminate crossbreeding. Though Jamunapari is the most popular breed in India, the purebred population has a decreasing trend over the decades (Singh and Chauhan, 2021).
- Lack of access to credit and insurance, these are common denominators in the different smallholders productions systems in India.

9. Prospects of dairy goat farming in India

To address the various challenges, the way forward will necessitate a wider recognition, better resource use, strong interdisciplinary approaches and institutional support to ensure the future contribution of goats in India as a dairy animal. In addition, promotion of goat milk consumption and creation of marketing support facilities are needed. Because the goat farmers are mostly unorganized and scattered and belong to the lower strata of the society, it is imperative to streamline the goat milk collection by establishing milk sheds in the home tract regions of the different breeds of dairy goats especially in the states of Uttar Pradesh, Rajasthan and Gujarat. This can be achieved through establishment of goat producers/breeders co-operative societies in the line of milk producer's co-operative societies established under Operation Flood projects (i.e. Anand Pattern of cows and buffalo milk marketing system – see below). Such efforts will ensure supply of goat milk in bulk for industrial use, and also enhance the production of healthy goat milk and milk products.

The "Anand Pattern of cows and buffalo milk marketing system" is an integrated cooperative structure that procures, processes and markets produce. Supported by professional management, producers decide their own business policies,

adopt modern production and marketing techniques, and receive services that they can individually neither afford nor manage. The Anand Pattern succeeds because it involves people in their own development through cooperatives where professionals are accountable to leaders elected by producers. The institutional infrastructure – village cooperative, dairy and cattle feed plants, state and national marketing – is owned and controlled by farmers. Anand Pattern cooperatives have progressively linked producers directly with consumers. It comprises a three-tier structure:

- **The Village Society:** An Anand Pattern village dairy cooperative society (DCS) is formed by milk producers. Any producer can become a DCS member by buying a share and committing to sell milk only to the society. Each DCS has a milk collection centre where members take milk every day. Each member's milk is tested for quality with payments based on the percentage of fat and SNF. At the end of each year, a portion of the DCS profits is used to pay each member a patronage bonus based on the quantity of milk produced.
- **The District Union:** A District Cooperative Milk Producers' Union is owned by dairy cooperative societies. The Union buys all the societies' milk, then processes and markets fluid milk and products. Most Unions also provide a range of inputs and services to DCSs and their members (e.g. feed, veterinary care, artificial insemination) to sustain the growth of milk production and the cooperatives' business. Union staff train and provide consulting services to support DCS leaders and staff.
- **The State Federation:** The cooperative milk producers' unions in a state form a State Federation, which is responsible for marketing the fluid milk and products of member unions. Some federations also manufacture feed and support other union activities (Shah, 1987).

Dairy goat farming in India has potential and the commercial goat farming units are established under intensive/semi-intensive system of management with dual purpose/dairy breeds of goats in different parts of the country. There are many potential advantages of starting commercial dairy goat farming business in India, and the main advantages are: (i) goat milk is a great source to fulfil the daily family nutritional demand; (ii) setting up a dairy goat farming business is much easier than for a cattle or buffalo dairy farm, requiring less initial investment capital; and (iii) goat milk products have a great demand in the local and international markets. The demand is increasing day-by-day and commercial dairy goat farming can be a great source of employment for the unemployed educated youth and rural women (NAP, 2017). After utilizing their milk producing potential, the goats could be sold for slaughter purposes, which is another source of income. The dairy goat sector in India requires a systems approach whereby nutrition, animal health, breeding, know-how, inputs and technologies are used to optimize natural and local resources used. There is an urgent need to develop strategies regarding breed conservation, management, health care, credit, insurance and marketing systems for the goat industry. The future of dairy goat breeds in India lies in implementing appropriate approaches to conservation, combining a number of integrally related components and effective action programmes approached holistically for successful conservation of dairy goat genetic resources.

In India, though commercial goat farming under intensive and semi-intensive systems of management has been picking up for the past couple of years, only less than one percent of goat population in the country has come under such a production system (Thiruvenkadan, 2012; NAP, 2017; Report, 2019b). There have been some efforts only by individual entrepreneurs to develop this enterprise besides R&D efforts of a few NGOs. Of late, some state governments have started making efforts towards promoting goat rearing (NAP, 2017). Goat rearing, which was the economic activity of rural resource-poor people, has attracted large and progressive farmers, businessmen and industrialists due to its economic viability under intensive as well as semi-intensive systems of management for commercial production. The entry of resource-rich people, including poultry farmers, who have better access to technical knowledge, resources and markets, into this activity would help in realizing the potential of this enterprise (Thiruvenkadan and Chidananda, 2018). Recent initiatives across the developing world emphasize goat-oriented activities as pathways out of poverty. Government bodies and development agencies such as the Doubling Farmers Income Committee have emphasized small ruminants as important contributors to poverty alleviation (Chand, 2017). In addition, goat sector development is an important component of the National Rural Livelihood Mission. and ongoing projects of the UN agencies and World Bank include goat sector development as a key project component (Escareño *et al.*, 2013; Lu, 2017b; NAP, 2017; Miller and Lu, 2019). The European model shows that the dairy goat sector can be modern, sanitary and profitable, with high quality products and global markets. The professionalization of dairy goat producers in France was supported by Government Agricultural Policy, which included facilitation of strong and trustworthy producers' unions (Lu, 2017b; Miller and Lu, 2019). In India, national policies must be developed with the participation of all actors in the field because they determine who will benefit from the growing market. Community-based and farmer-group approaches offer opportunities to improve livestock genetics and the quick adaptation of new technologies. The use of participatory research projects is recommended in addressing the needs of smallholders and should be followed-up with on-farm research projects (Thiruvenkadan and Chidananda, 2018).

Several reports suggest there is a growing niche market in India for goat milk for Ayurveda medicinal preparations, cheese production and export for value-added products as it leads to better digestibility, higher availability of micronutrients, presence of viable lactic bacteria and production of certain bioactive peptides and other health benefits (Thiruvenkadan, 2012; Thiruvenkadan and Rajendran, 2014; NAP, 2017; Thiruvenkadan and Chidananda, 2018). Processing goat milk into high-value products, particularly goat cheese, may be one way to expand dairy goat farming in India. Indian goat cheese could possibly compete in the international market as well. In order to achieve long-term success, the French approach (Lu, 2017b; Miller and Lu, 2019; Liang and Paengkoum, 2019; Lu and Miller, 2019) which focuses on governmental support, improving marketing systems, responding to changing consumer demand and using more technology provides a model to be followed. Since goat producers in India are poor (NAP, 2017) and thus do not have enough resources to solve their challenges, or to apply impact technologies, they need adequate policies and investment by governments and development agencies. As the global dairy goat industry continues to integrate through sharing resources and for improving the product as well as processing methodologies, the challenge is to facilitate subsistence producers' transition to commercialization by understanding and addressing their motivations and obstacles. The growing global market for dairy goat products can be good for producers and consumers of all countries, including India. Competition can lead to innovation, improved quality and greater choice for consumers. Increasing milk yields for direct consumption and for processing into butter, yoghurt and cheeses are major selection aim in the development of better dairy goats in Europe and America (Escareño *et al.*, 2013; Lu, 2017b; Miller and Lu, 2019; Liang and Paengkoum, 2019). Increasing cheese and yogurt yields without increasing milk yields led to the development of dairy sheep with high contents of total solids in their milk, contrary to the evolution in dairy goat breeds. Government involvement has been significant in sanitary regulation, research, extension, support for local producer organizations, and markets, and ensures safety and quality in Europe and America (Devendra and Liang, 2012; Shinde and Naqvi, 2015; Thiruvenkadan and Chidananda, 2018; Liang and Paengkoum, 2019).

10. Conclusions

The dairy goat industry in India has been slowly adopting science and technology. The main marketing limitation for prospective dairy goat producers is the small number of commercial processors to whom raw milk can be shipped. However, dairy goat production in India is an alternative livestock enterprise suitable for many small-scale or part-time livestock operations. In order to increase the consumer preference for goat milk consumption, it is therefore necessary to attempt a realistic appraisal of the overall situation, taking into account the special attributes of goat's milk, assessing the milk production potential of various breeds, and considering the efficiency with which it is produced in comparison with other lactating ruminants. To very resource-poor peasant farmers in India, rearing of dairy goats is better than cattle because of its faster generation turnover and earlier milk production. The goat probably will never replace the cow for commercial production of milk, but there seems to be a great potential for diligent efforts in practice and research to improve production and marketing of goat milk and its products. Developing the sector will require focused efforts on up-gradation of milch breeds, support to commercial farms, encouraging entrepreneurs to invest in the sector through training programmes on processing along with facilitating access to finance.

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The effect of partial replacement of concentrate by browse species *in vitro* and on growth performance of growing goats

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Abstract

A sustainable alternative to mitigate the high price of concentrate feeds for ruminants is to produce good quality fodder to replace part of the purchased concentrate in the diet. The utilization of locally available and home-grown high protein forages and browses is therefore being encouraged. The objective of this study was to investigate the *in vitro* rumen fermentation kinetics and dry matter intake and growth of goats fed a partial replacement of concentrate by browse species, "Bois Noir" (*Albizia lebbeck; AL*) and "Gros Feuilles" (*Litsea monopetala*; *LM*). The selected feeds sugarcane tops (*Saccharum officinarum*; *SCT*), *AL*, *LM* and concentrate (compounded feed with 17 percent of crude protein; CP) were evaluated using the *in vitro* gas production technique. Eighteen crossbred goats weighing on average 19.3 ± 4 kg were divided into three groups in a completely randomized design and allocated to different treatments. The control group of animals (CG) received sugarcane tops and locally manufactured concentrate in a ratio of 60:40 on dry matter basis. The Treatment 1 (TAL) animals received fodder, concentrate and *AL* in a ratio of 60:20:20. For Treatment 3 (TLM), the animals received fodder, concentrate and *LM* in a ratio of 60:20:20. *In vitro* study showed that upon replacing 50 percent of concentrate by browse species, the gas production was 38 and 36 mL/200 mg for *AL* and *LM*, respectively, after 72 h of incubation, which was not different from that with concentrate only. After 136 days, the average live weights for the animals in CG were 24.6 kg compared to 22.5 kg and 22.5 kg for TAL and TLM respectively. There was no significant difference ($P < 0.05$) in live weight among the treatment groups. The average daily gain (ADG) for the CG of animals was 30 ± 6 g/day compared to 28 ± 2 g/day and 25 ± 7 g/day for TAL and TLM, respectively. There was no significant difference ($P < 0.05$) in the ADG among the treatment's groups. This study provides estimates of the nutritive value of the browse species and suggest potential for replacing 50 percent of concentrates in the diet with browse species such as *AL* and *LM*.

Keywords

goats, browse species *Albizia lebbeck* and *Litsea monopetala*

1. Introduction

Animal feeds and feeding are of critical importance in livestock production systems. They have impacts on animal productivity, health and welfare, product quality and safety, producer incomes, household security, land use and land use change, water pollution and greenhouse gas emission (FAO, 2012). Most smallholder farmers in Mauritius use the cut and carry system for collecting fodder intended for feeding their animals (Boodoo *et al.*, 1999). Tree and browse species are important components of ruminant diets and are rich in most essential nutrients, particularly proteins and minerals (Saraye *et al.*, 2016). Although they are important sources of forage throughout the year for smallholder farms, little information is available on their nutritional value locally. A sustainable alternative to mitigate the high price of concentrate feeds is to produce good quality fodder (grass and leguminous species) to replace part of the concentrate in the diet of ruminants. The utilization of locally available and home-grown high protein forages and browses is therefore being encouraged in Mauritius. Two local browse/tree species found abundantly in abandoned and forest lands are *Albizia lebbeck* (*AL*) and *Litsea monopetala* (*LM*).

Albizia lebbeck (Figure 1) is a nitrogen-fixing tree, proposed as an alternative forage to *Leucaena leucocephala* in acid soils, particularly with the advent of the leucaena psyllid (*Heteropsylla cubana*). Green leaf has a 16–23 percent of crude protein (CP) and has shown to be highly palatable and of high nutritive value for ruminants (Tropical Forages, 2022).

Figure 1: "Bois Noir" (*Albizia lebbeck*; *AL*)

Litsea monopetala (*LM*) is an important evergreen fodder tree. It has a fast growth with high foliage production with a good nutritional value of leaves with CP of 14–18 percent(Shrestha, 2017).

Figure 2: "Gros Feuilles" (*Litsea monopetala*; *LM*)

The objective of this study was to investigate the *in vitro* rumen fermentation kinetics, dry matter intake (DMI) and growth of goats fed of partial replacement of concentrate by the browse species *AL* and *LM*.

2. Materials and methods

All applicable national and international guidelines for care and use of animals were followed while carrying out this experiment.

In vitro studies

Rumen fluid was obtained from two fistulated bulls fed twice daily with a diet containing sugarcane tops (*Saccharum officinarum*; SCT; 60 percent) and compounded feed with 17 percent of CP (40 percent). The selected feeds SCT, *AL*, *LM* and concentrate (compounded feed with 17 percent of CP) were incubated using calibrated glass syringes (FORTUNA Haberle Labortechnik) following the procedures of Menke and Steingass (1988). Dried (60 °C) and ground (1 mm) samples of approximately 200 mg of DM were weighed in triplicate into glass syringes. The samples were incubated alone and in mixture. For the basal diet, *SCT* were mixed with concentrate at a DM ratio of 60:40. Diets were also formulated to replace 50 percent of the concentrate with each of the browse species such that the ratio of *SCT*: concentrate: browse species was 60:20:20.

The syringes were pre-warmed at 39 °C in an incubator before the injection of 30 ml of a rumen fluid-buffer mixture into each syringe followed by incubation in a water bath at 39 °C. The syringes were gently shaken 30 min after the start of incubation and every hour for the first 6 hours of incubation. Gas production was recorded before incubation (0 h) and thereafter at 2, 4, 6, 24, 48 and 72 h after initiation of incubation. Total gas values were corrected for blank and hay standards of known gas production. Cumulative gas production data were fitted to the model of Ørskov (2000) using the Curve Fit Neway computer program (X.B. Chen, Rowett Research Institute, Aberdeen).

$$p = a + b\,(1\text{-}\exp^{-c\,t})$$

Where:

- p represents gas volume (ml) at time t;
- a is the gas produced from soluble fraction (ml);
- b is the gas produced from insoluble but fermentable fraction (ml);
- $(a+b)$ is the potential gas production (ml); and
- c is the rate constant of gas production during incubation (ml h^{-1}).

Feeding Trial

The experiment was conducted at the Curepipe Livestock Research Station, Agricultural Research and Extension Unit, Mauritius.

Feeding

The animals were fed as per the diets given in Table 1. The CG animals received sugarcane tops and locally manufactured concentrate in an approximate ratio of 60:40 on a dry matter basis, whereas in the Treatment 1 (TAL) the animals received fodder, concentrate and *AL* in a ratio of 60:20:20, and in Treatment 2 (TLM), the animals received fodder, concentrate and *LM* in a ratio of 60:20:20.

Table 1: Diets composition on fresh and dry matter basis

Treatments	CG		TAL and TLM	
	Fresh matter	Dry matter	Fresh matter	Dry matter
Fodder, kg/day	3.00	**0.75**	3.00	**0.75**
Concentrate, kg/day	0.50	0.40	0.25	0.20
Browse species, kg/day	0.00	**0.00**	1.00	0.20
Total, kg/day	**3.50**	**1.15**	**4.25**	**1.15**

Source: Authors' own elaboration.

Fodder

The fodder was chopped, 10–15 cm long, before giving to the animals. For the browse's species, only the leaves and the young twigs were fed, and hard wood was removed.

Animals, diets and experimental design

Eighteen crossbred goats weighing on an average 19.3 ± 4 kg were divided into three groups in a completely randomized design and allocated to different treatments. Six animals, 3 males and 3 females, were allocated to CG, TAL and TLM, respectively. The animals were grouped and housed according to sex and treatments to avoid bullying, and had continuous access to fresh water throughout the duration of the experiment The animals were allowed to adapt for 15 days prior to the start of the experimental period and treated against internal and external parasites using Invermectin.

The daily allowance of the feeds was given in two equal meals at 8.30 h and 14.30 h respectively. Mineral was included in the concentrate fed to the animals. Twice weekly the intake and residual diets were weighed for each group of animals, and representative samples collected for proximate analysis. The live weight was recorded fortnightly and feed intake was monitored daily.

Parameters monitored were:

- Weight at start of experiment and every fortnightly.
- Daily feed intake (fodder type and concentrates).
- Samples of all feeds were taken for proximate analysis.

The experiment lasted for 136 days.

Chemical analysis

Samples of all feeds were periodically determined for CP, crude fibre (CF), dry matter (DM), ether extract (EE) and ash according to Association of Official Analytical Chemists procedures (AOAC, 1990). The gross energy (GE) was calculated using appropriate equations from the MAFF Technical Bulletin No. 33 (MAFF, 1975).

Data were analysed using the MIXED procedure of SAS (2003). The model included the fixed effects of period and treatment.

3. Results and discussion

The chemical composition of the feeds and mixture of fodder browses and concentrate is shown in Table 2.

Saccharum officinarum had 5.6 ± 3.5 percent of CP while *AL* and *LM* had 15.5 ± 2.5 percent, and 15.6 ± 1.35 percent of CP, respectively. The figures are in accordance with that found in the literature (Feedipedia, 2012; Tropical Forages, 2022). Because of greater CP levels in the browse species compared to the SCT, a mixture of fodder and browse can be used to meet animal requirements. The diet prepared by replacing 50 percent of the concentrate by browse species resulted in a CP of the diet of about 9–10 percent, which was comparable to that of using concentrate only (with SCT).

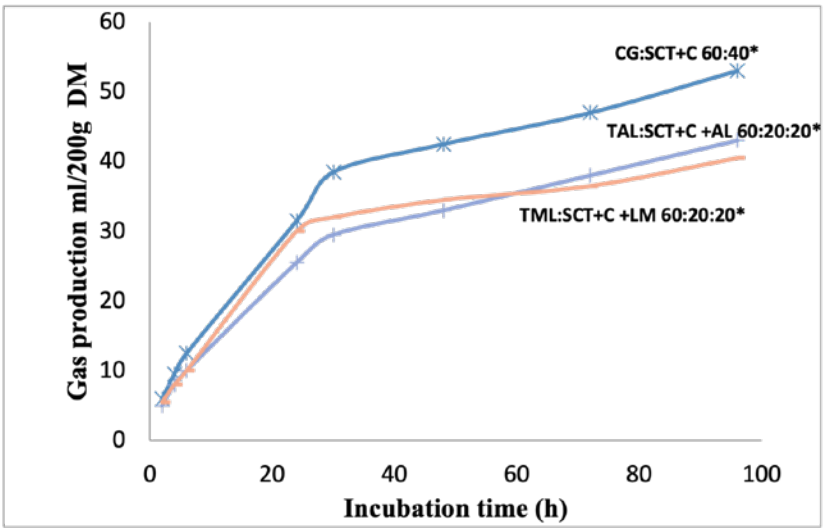
Table 2: Chemical composition of the feed

Feeds*	DM (%)	% DM								
		CP	CF	EE	ASH	P	Ca	ADF	ADL	NDF
SCT	33.5	5.6	34.7	1.7	5.4	0.3	0.2	41.8	5.9	70.6
AL	42.0	15.5	28.1	4.0	8.2	0.3	1.7	34.3	11.8	46.1
LM	54.1	15.9	29.2	3.8	5.4	0.3	0.8	35.1	11.2	47.9
C	86.5	17.2	4.2	3.8	6.4	1.4	1.2	-	-	-
CG	54.1	10.5	22.4	2.4	6.6	0.7	0.6	25.1	3.5	42.4
TAL	45.4	9.2	26.4	2.8	7.1	0.5	0.8	31.0	5.4	50.4
TLM	42.5	9.9	27.7	2.2	6.3	0.6	0.5	34.9	7.6	53.0

Source: Authors' own elaboration.

Note: * SCT: sugar cane tops; *AL*: *Albizia lebbeck*; *LM*: *Litsea monopetala*; C: Concentrate; CG: SCT+C (60:40); AL: SCT+C+*AL* (60:20:20); TML: SCT+C+*TM* (60:20:20); DM: dry matter, CP: Crude protein, CF: Crude Fiber, EE: Ether extract, P: Phosphorous, Ca: Calcium, ADF: Acid Detergent Fibre, ADL: Acid Detergent Lignin, NDF: Neutral Detergent Fibre.

The *in vitro* cumulative gas production (mL/200mg DM) of the SCT, concentrate, and different combinations with *AL* and *LM* are shown in Figure 3. The basal diet resulted in greater gas production (47 ml/200mg DM) after 72 h of incubation. Upon replacing 50 percent of concentrate in the ratio of 60:20:20 (SCT: concentrate: browse species) gas production was 38, and 36 mL/200 mg for mixtures including *AL* and *LM*, respectively, after 72 h of incubation. Greater gas production indicates greater DM degradability for the basal diet mixed with concentrate.



Note: *CG: SCT+C (60:40); AL: SCT+C+*AL* (60:20:20); TML: SCT+C+*LM* (60:20:20).
Source: Author's own elaboration.

Figure 3: *In vitro* analysis of fodder and feed mixture (CG = control group; SCT = sugarcane tops; C = concentrate; *AL* = *Albizia lebbeck*; *LM* = *Litsea monopetala*)

The results of the feeding trial are shown in Table 3. The average initial body weight (BW) for CG animals was 20.3 kg compared to 18.7 kg and 18.9 kg for TAL and TLM, respectively. The average final BW for CG was 23.8 kg compared to 22.0 kg and 21.4 kg for TAL and TLM, respectively.

No treatment difference ($P < 0.05$) was observed on BW and average daily gain among the three diets. Thus, the feeding experiment with goats concluded that *AL* and *LM* have potential as leaf meal-based supplements to partially replace purchased concentrate as protein sources for growing goats fed a poor quality basal diet.

Table 3: Results of feeding trial

Diet		No. of animals	Initial average body weight, kg	Final average body weight, kg	Average daily gain, g/day
CG	SCT: C*	3 males	18.2 ± 2.3	22.3 ± 3.5	40 ± 3
		3 females	22.5 ± 5.2	26.7 ± 4.8	30 ± 5
TAL	SCT:C:*AL*	3 males	16.7 ± 3.1	22.0 ± 4.5	40 ± 2
		3 females	20.7 ± 7.1	23.0 ±7.9	17 ± 5
TLM	SCT:C: *LM*	3 males	17.8 ±1.3	20.8 ± 0.1	22 ± 7
		3 females	20.0 ± 4	24.0 ± 3.4	30 ± 7

Source: Authors' own elaboration.
Note: * SCT: sugarcane tops, (*Saccharum officinarum*) C: Concentrate, *AL*: *Albizia lebbeck*, *LM*: *Litsea monopetala*.

4. Conclusions

The results obtained in this study include estimates of nutritive values of *AL* and *LM* and show potential for replacing 50 percent of concentrates in the diet with browse species. Because our numbers were small, repetition of the study is warranted. The results favoured the diets a full complement of concentrates, but the differences were not statistically significant at the level of power available with 18 animals. With renewed interest in using shrubs/trees in ruminant feeding as a means to reduce use of costly concentrate, more research is required for optimal introduction of these feed resources in feeding systems.

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Genetic variability among indigenous cattle populations in Sri Lanka

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Abstract

The present study was designed to perform the genetic characterization of six indigenous cattle populations (two distinct populations of Thamankaduwa – Th, Thawalam – TW, North Central – NC, Southern – So and Northern – No) in Sri Lanka. A total of 189 samples were genotyped using 11 autosomal microsatellite DNA markers, of which 121 alleles were detected, indicating a high allelic diversity. The overall mean number of alleles per marker per population was 11 varying from 7.27 (Th) to 8.36 (No). The observed and expected heterozygosities per locus were 0.745 and 0.797, respectively. The mean inbreeding coefficient (F_{IS}) was 0.043 (ranging from 0.020 in Th to 0.073 in TW). All six populations showed a considerable level of inbreeding and the highest heterozygosity deficit, in the TW population, was clearly explained by its breeding management. Only 3.4 percent of the total genetic variation (F_{ST} = 0.034) was between-populations. The pairwise Cavalli-Sforza and Edwards chord distances between the six populations clearly showed that TW cattle, which are pack animals with specific body conformation traits, were separated from other populations. However, there was no apparent discrete genetic structure among these six populations.

Keywords

genetic diversity, microsatellite DNA marker, indigenous cattle, Sri Lanka

1. Introduction

Cattle farming has been observed in Sri Lanka from ancient times. In 2020, the total cattle population was 1.1 million in Sri Lanka, which are mostly distributed in the dry zone of the country and are reared under rural low input–low output systems (Silva *et al.*, 2010; Vithanage *et al.*, 2013; Department of Census and Statistics, Sri Lanka, 2020). The current Sri Lankan cattle population counts mainly three breed types; namely, the indigenous, the exotic and the crossbred. Indigenous breeds have been always identified as important animal genetic resources that have not been well described to date (Silva *et al.*, 2010). Indigenous breeds in Sri Lanka include common Lankan Cattle or "Batu Harak" (*Bos indicus var ceylonicus*), Thamankaduwa cattle (also known as Thamankaduwa white or white cattle) and Thawalam cattle or pack animals. The Lankan breed can be found in multiple geographical areas of the country; whereas the Thamankaduwa and the Thawalam cattle are limited to certain areas of the country. Thamankaduwa cattle are restricted to the Eastern Province of Sri Lanka. Thawalam cattle are used as pack animals in the central hills of Sri Lanka; therefore they are geographically isolated and phenotypically different from the other cattle populations. Indigenous breeds are known to be mostly well adapted to local harsh environmental conditions, however, their production performance is relatively low compared to the exotic or crossbred breeds (Wijeweera *et al.*, 2014; Silva *et al.*, 2021). To this extend, crossbreeding of the indigenous breeds with different improved exotic breeds, has become a

common practice for decades (Silva, 2010). Unfortunately, the implemented crossbreeding programmes were not defined in accordance to a well-studied selection procedure, resulting in a considerable loss of some important genetic features such as diease resistance and adaptability to the environmental conditions of the Sri Lankan indigenous cattle populations (Wijeweera *et al.*, 2014; Samaraweera *et al.*, 2020; Silva *et al.*, 2021). Following such a situation in Sri Lanka and other countries, and highlighting the importance of animal genetic resources, FAO (2007) brought global attention on their conservation. To this extent, relevant genetic identification and characterization of local animal genetic resources are fundamental for the establishment of successful conservation plans. Up to now, studies made on indigenous cattle populations in Sri Lanka have exclusively focused on their phenotypic characterization, their production features as well as on their farming systems (Abeygunawardena, Rathnayaka and Jayathilaka, 1997; Silva *et al.*, 2010; Wijeweera *et al.*, 2014; Shanjayan and Lokugalappatti, 2015). To date, only one study has reported an attempt on genetic characterization of some populations of indigenous cattle in Sri Lanka; North Central, Southern, Northern cattle populations and the Thamankaduwa cattle population (Silva *et al.*, 2010). The molecular investigations of this study revealed a high level of genetic diversity within populations and a predominant Zebu origin in Southern, Northern and Thamankaduwa cattle populations with subsequent introgression of taurine genetics in the North Central cattle population.

No attention has been reported to the genetic characterization of Thawalam cattle (pack animals), which are the unique draught cattle reared in isolated pockets of Sri Lanka. The present study intended to revisit the results generated by the same research group (Silva *et al.*, 2010). and fill in missing data by conducting a more extensive genetic analysis of six geographically distinct indigenous cattle populations in Sri Lanka.

2. Materials and methods

Sample collection

A total of 49 blood samples were collected from two local cattle breeds, namely the Thamankaduwa cattle (Th) and the Thawalam cattle (TW) from the Eastern Province and the Central part of Sri Lanka, respectively. Among which, 22 samples came from the Th herds located in the Ampara District of the Eastern Province and 27 samples come from the TW herds of the Badulla District within the Uva Province and Hunnasgiriya in central hilly area of Sri Lanka. Masterpure® DNA extraction kits were used to extract DNA from the collected blood samples. Additionally, 16 DNA samples from a previous study conducted by Silva *et al.* (2010) were added to the newly extracted ones with the aim to obtain an wide view of the genetic architecture of indigenous cattle population of Sri Lanka. These 16 samples were selected as follows: four from the North Central (NC) cattle population, four from the Southern (So) cattle population, four from the Northern (No) cattle population and four from the Thamakaduwa 1 cattle populationa (Th1; from the northern boundary of Eastern Province).

Microsatellite genotyping

DNA genotyping was conducted at the Animal Genetic Resources Research Center, National Institute of Animal Science, Hamyang, Republic of Korea.

DNA samples were subjected to polymerase chain reaction (PCR) amplifications of 11 microsatellite DNA markers, namely BM2113, CSSM66, ILSTS006, MGTG4B, TGLA122, HEL1, AGLA293, ILST005, INRA035, ILSTS050 and ILSTS023. Markers were selected in accordance with the recommended microsatellites by the International Society for Animal Genetics and the Food and Agriculture Organization of the UN (FAO, 2011). The same set of markers was used previously in the study of Silva *et al.* (2010). The PCR was performed in a total reaction volume of 25 µL using the following thermal conditions: 94 °C for 10 minutes, followed by 35 cycles of 94 °C for 45 seconds, specific annealing temperature of 55 °C – 60 °C for 45 seconds and 72 °C for 45 seconds and a final extension at 72 °C for 10 minutes. The PCR amplification was confirmed by agarose gel electrophoresis. The PCR products were genotyped using a capillary sequencer (ABI 3730 DNA Analyzer – Applied Biosystems). The electropherogram analysis for allele size determination was carried out using GENEMAPPER software (Applied Biosystems).

Genotyping of the 16 samples from Silva *et al.* (2010) along with 49 DNA samples from present study facilitated merging of the genetic information from the complete collection of 140 samples from the previous study of Silva *et al.* (2010), which consisted of NC (38), So (28), No (37) and Th1 (37) cattle populations. The data sets from Silva *et al.* (2010) and from the current study were aligned for allele sizes and merged for the final analysis. The allele size received by genotyping 16 samples from Silva *et al.* (2010) were used as the baseline for aligning of allele size of previous data set with the current data set and aligning has been carried out manually. The final data set consisted with genotypes derived from a total of 189 cattle. Although Thamankaduwa cattle from both studies are phenotypically similar, Thamakaduwa (Th and Th1) samples from two studies were considered as two cattle populations as they were from two separate locations. Further, by considering Thamankaduwa cattle as Th and Th1, we accounted for any time effect on the genetic diversity and population genetic structure of the overall Thamankaduwa population. Thus, a total of six cattle

populations: including Th, TW, NC, So, No and Th1, were assessed for their genetic diversity and population genetic structure.

Data analysis

The calculated genetic parameters included the observed number of alleles, the observed and the expected heterozygosity and the fixation index (F_{st}). These parameters were calculated using MICROSATELLITE ANALYZER software version 4.05 (Dieringer and Schlötterer, 2003). Using GENEPOP software version 4.1.3 (Raymond and Rousset, 1995), deviation from the Hardy-Weinberg equilibrium (HWE) was identified by estimating the rate of heterozygosity reduction within-population, due to inbreeding (F_{IS}), together with the heterozygote excess and deficit for each marker and each population.

A phylogenetic tree was built on the basis of pairwise Cavalli-Sforza and Edwards chord genetic distances among the sampled group of animals. The unweighted pair-group method was applied using arithmetic averages (UPGMA) algorithm using PHYLIP version 3.5 (Felsenstein, 1993).

Principal component analysis (PCA) was carried out using SPSS software version 13.0 with pairwise F_{ST} derived from allele frequencies. The population structure and the levels of individual admixture were determined by Bayesian clustering analysis of STRUCTURE version 2.3.4 (Pritchard, Stephens and Donnelly, 2000) with a cluster assumption (K) deriving from 2 to 6. For each K, 30 replicates were run. The length of burn-in periods and number of Markov Chain Monte Carlo iterations used for all the runs were 100 000 for each. The most probable number of clusters (K) was selected based on highest average log probability of data with the lowest variation among the 30 runs. Using the web-based STRUCTURE HARVESTER software (Earl and von Holdt, 2012), Ad hoc quantity (ΔK) was calculated to verify the best K, in accordance to the fluctuation rate of log probability of data between the successive K values. The results were post-processed using CLUMPP software version 1.1.2 (Jakobsson and Rosenberg, 2007) and plots were generated using Microsoft Office Excel 2010.

3. Results and discussion

Merging of genetic information from Silva *et al.* (2010) and the present study helped to view comprehensive information on the genetic diversity and population genetic structure in six different cattle populations: including Th, TW, NC, So, No and Th1, in Sri Lanka.

A total of 121 alleles were detected for the six cattle populations, indicating a high allelic diversity in indigenous cattle in Sri Lanka. The mean number of alleles per locus was 11. The highest number of alleles was 15 for the locus CSSM66, while the lowest number was six for the locus ILST005. Within populations, the numbers of alleles were relatively high and varied from 7.27 (Th) to 8.36 (No), reflecting an important allelic diversity between the studied cattle populations. These findings overlapped with those observed in Ongole, Maweti and Ponwor cattle breeds of the Indian (Sharma *et al.*, 2015) and Dhani, Lohani, Cholistani breeds of the Pakistani (Hussain *et al.*, 2016) cattle populations.

However, allelic diversity measures of Sri Lankan cattle populations were higher than some indigenous cattle breeds, such as Dehoni, Hariana, Sahiwal and Kherigarh cattle reported in India (Mukesh *et al.*, 2004; Pandey *et al.*, 2006), Therparker and Red Sindi of the Pakistan (Hussain *et al.*, 2016) and Creole cattle (Delgado *et al.*, 2012) or Brown Swiss (Schmid *et al.*, 1999) from Europe, but lower than Algerian local breeds (Rahal *et al.*, 2021). The high allelic diversity observed in Sri Lankan cattle populations may be a result of an absence of directional selection pressure. Indigenous cattle are generally reared in extensive production systems where natural breeding occurs with negligible selection pressure.

The overall mean of the observed heterozygosity per locus was 0.745, ranging between 0.556 and 0.882, respectively, for the loci ILSTS023 and TGLA122. The mean of the expected heterozygosity per locus was 0.797 ranging between 0.605 and 0.871, respectively, for the loci HEL1 and TGLA122.

The genetic variability was observed to be similar across the studied Sri Lankan cattle populations. Among populations, the mean observed heterozygosity varied between 0.716 (TW) and 0.765 (Th1) and the mean expected heterozygosity ranged from 0.755 (So) to 0.785 (Th1). The allelic diversity, observed and expected heterozygosity and inbreeding coefficients of the six Sri Lankan cattle populations are summarized in Table 1.

The inbreeding coefficient (F_{IS}) varied between 0.020 (Th) and 0.073 (TW) with an overall mean of 0.043.

The HWE test revealed significant deviations ($P < 0.05$) from the equilibrium in 13 out of the 66 breed-locus combinations. Five loci in NC (BM2113, ILSTS006, MGTG4B, ILSTS023 and ILSTS050), two loci each in Th (INRA035 and ILSTS023), TW (ILST005 and INRA035) and So (BM2113 and ILSTS023), and one locus each in Th1 (ILSTS023) and No (ILSTS023) cattle populations deviated from HWE (Table 2). Among the different loci, ILSTS023 significantly deviated from HWE in five out of the six studied cattle populations. Furthermore, all these deviations were due to heterozygosity deficit within the populations. Holsinger and Weir (2009) described that F_{IS} estimators are relevant indicators for the adopted mating system within populations. Accordingly, the observed heterozygosity deficits in all populations reflect a high level of inbreeding. The highest heterozygosity deficit, observed in the TW cattle population, could be explained

Table 1: Genetic diversity of indigenous Sri Lankan cattle populations

Parameter	Thamankaduwa cattle population	Thamankaduwa cattle1 population	Thawalam cattle population	North Central population	Southern population	Northern population
n_o	7.2	7.7	7.4	7.7	8.0	8.4
H_o	0.753	0.765	0.716	0.749	0.738	0.736
H_e	0.768	0.785	0.771	0.783	0.755	0.775
F_{IS}	0.020	0.030	0.073	0.050	0.026	0.062

Source: Authors' own elaboration.
Note: n_0 – Mean number of alleles per population, H_o – Observed Heterozygosity, H_e – Expected Heterozygosity, F_{IS} – Inbreeding Coefficient.

Table 2: Hardy-Weinberg equilibrium at different microsatellite loci in Sri Lankan cattle populations

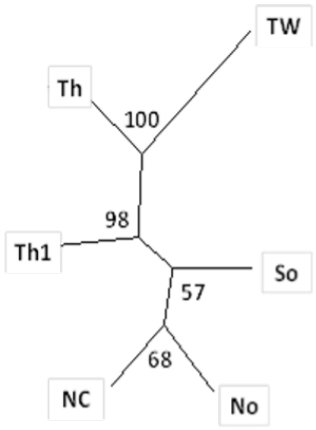
	Thamankaduwa cattle population		Thawalam cattle population		Thamankaduwa cattle 1 popultion		North Central population		Sothern population		Northern population	
	*F*IS	P value	*F*IS	P value	*F*IS	P value	*F*IS	P value	*F*IS	P value	*F*IS	P value
BM2113	0.0291	0.533	0.0359	0.979	0.0372	0.841	0.035	0.023	0.1942	0.000	-0.0385	0.889
CSSM66	-0.0390	0.923	0.2291	0.229	0.0060	0.697	0.007	0.491	0.0410	0.055	-0.0709	0.406
ILSTS006	-0.0983	0.223	-0.0656	0.718	0.0133	0.779	0.200	0.001	0.1845	0.069	0.1892	0.202
MGTG4B	-0.1688	0.928	-0.2815	0.340	0.0495	0.754	0.148	0.000	-0.0769	0.090	0.0064	0.381
TGLA122	-0.1396	0.136	0.0371	0.392	0.0641	0.118	0.024	0.132	-0.1103	0.688	0.0633	0.117
HEL1	-0.0856	0.877	0.3310	0.053	-0.0854	0.269	-0.146	0.728	-0.0725	0.871	0.0568	0.451
AGLA293	-0.0730	0.964	-0.0827	0.567	0.0116	0.251	0.020	0.710	-0.0659	0.908	0.0932	0.069
ILST005	-0.0319	0.488	0.0339	0.001	-0.0362	0.182	0.015	0.786	0.1195	0.171	0.0793	0.082
INRA035	0.2547	0.035	0.2358	0.001	0.0398	0.462	-0.053	0.300	-0.0146	0.116	-0.0827	0.615
ILSTS050	0.1111	0.210	0.0342	0.451	-0.0849	0.614	0.122	0.038	-0.0418	0.585	-0.0241	0.658
ILSTS023	0.4286	0.029	0.2813	0.081	0.2835	0.000	0.166	0.027	0.1582	0.043	0.4702	0.000

Source: Authors' own elaboration.
Note: *F*is – Heterozygosity Deficit.

by the practiced breeding programmes according to which most of TW adult males are castrated and used as pack animals while a few "genetically superior" males are used for the breeding practices. These "genetic superior" males are usually identified and selected according to their body confirmation, which needs to be suitable for pack purpose (Kumaravithana, 2014). The long-term practice of selection of genetically superior animals for breeding while castrating the other males may have led to a small effective population size of the Thawalam cattle population, allowing for the high chance of inbreeding within the population. Further, the requirement of TW cattle population as a pack animal is gradually diminishing because of the improvement in physical environment and infrastructure facilities in and around farming systems in central hills of the country. Considering these external and internal pressures, the population of TW cattle may be facing a rapid genetic erosion. The limited number of farmers who still depended on the transportation using pack animals have been playing a champion role in conserving the genetic pool of TW cattle in Sri Lanka.

The global F_{ST} was 0.034, indicating that only 3.4 percent of the total genetic variation was due to between-population, while 96.6 percent of the variation is within the populations. As shown in Table 3, F_{ST} coefficients ranged between 0.0095 and 0.065 and the highest genetic differentation was observed between the TW population and the NC, So and No populations. This might be partially explained by the fact that the TW cattle population is geographically well separated from remaining populations. Figure 1 illustrates the genetic distances between the six studied cattle populations. The clustering of No, NC and So cattle populations together is consistent with their phenotypic similarity, all exhibiting the characteristics of Sri Lankan "Batu Harak" (Silva *et al.*, 2008). Although No, NC and So cattle populations are in different geographical locations, mixing of animals from these three populations is possible since these three areas are not geographically isolated. The genetic distance estimates clearly showed that TW cattle were separated from all other cattle populations with the exception of the Th cattle. The separation of TW cattle suggests a distinct genetic makeup, probably owing to their geographical

isolation and unique breeding management. Further, results showed that TW cattle are relatively closed to the Th cattle which had been co-sampled and genotyped during the current study. Phenotypically Th cattle are similar to Th1 cattle, although Th cattle were placed relatively close to TW, while Th1 were relatively close to NC, No and So in the present study. Hence, the results reflect clustering together of the cattle populations belonging to the two different datasets. This observed clustering pattern may have resulted from the time effect on the genetic diversity and population genetic structure of the Th population. However, possibility of an effect of merging the two datasets cannot be excluded since manual merging of microsatellite datasets that had been genotyped independently is error-prone due to differences in binning methods and molecular weight standards (Presson *et al.*, 2006).



Source: Author's own elaboration.

Figure 1: UPGMA Radial tree of six indigenous cattle populations; Thawalam cattle, Thamankaduwa cattle, Thamankaduwa cattle 1, Southern cattle, Northern cattle, North Central cattle populations in Sri Lanka

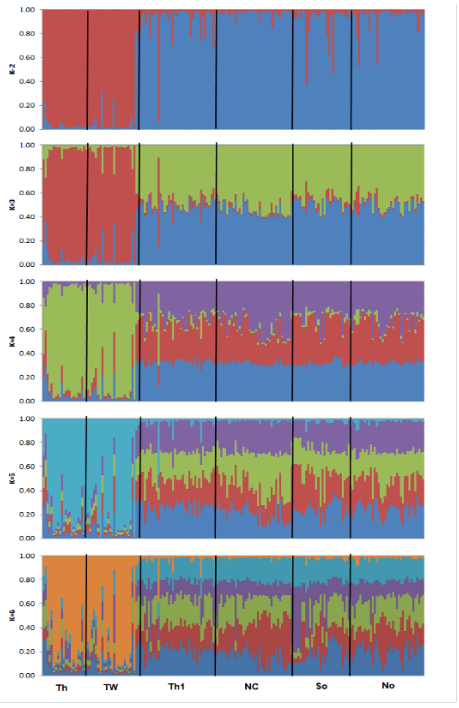
Table 3: F_{ST} estimates (upper triangle) and Cavalli-Sforza and Edwards chord distances (lower triangle) between six Sri Lankan cattle populations

	Th	TW	Th1	NC	So	No
Th	-	0.0096	0.0478	0.0650	0.0588	0.0610
TW	0.2773	-	0.0438	0.0599	0.0557	0.0608
Th1	0.3531	0.3391	-	0.0095	0.0191	0.0113
NC	0.3844	0.3800	0.2271	-	0.0245	0.0162
So	0.3588	0.3533	0.2381	0.2349	-	0.0172
No	0.3062	0.3771	0.2411	0.2340	0.2440	-

Source: Authors' own elaboration.

Note: Th – Thamankaduwa cattle population, Th1 – Thamankaduwa cattle 1 population, TW – Thawalam cattle population, NC – North Central cattle population, So – Southern cattle population, No – Northern cattle population.

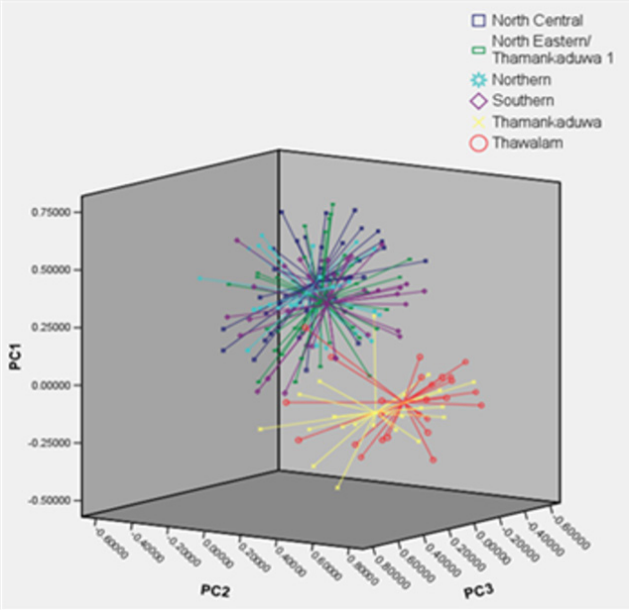
According to the structure analysis, $K = 2$ was the best K. A similar population genetic structure was observed among TW and Th individuals while the other four populations showed a separate common population structure (Figure 2). The PCA also showed clustering of TW and Th cattle, separated from the other four populations. In the present study, a total of 34 principal components with eigen values greater than one were extracted and they together explained more than 92 percent of the total variation in the dataset. The first-three principal components were plotted on a three-dimensional scattergram (Figure 3). The first, second and third principal components explained 12.21 percent, 8.21 percent and 7.47 percent of the total genetic variation, respectively. As discussed previously, the clustering of phenotypically similar Th cattle from the present study separately from the Th1 cattle of the previoyus study could be due to the time effect which may have changed the genetic structure of the Thamankaduwa population. The presence of few animals in the Th1 cattle population that have a genetic structure similar to the Th cattle is consistent with the possibility of a time effect on the observed differences between Th and Th1 cattle. However, the observation of clustering of Th cattle and TW cattle, for which all samples are from the present



Source: Author's own elaboration.

Figure 2: Bayesian clustering of 189 cattle under assumption of 2–6 clusters without a priori population information; the individuals of different populations separated by vertical black lines

study shows the potential for confounding between the two studies and possibility of allele miscalling between the two studies. The purpose of using microsatellite markers for the present study is to combine the genetic information from the previous study of Silva *et al.* (2010) with genetic information generated from some of the missing cattle populations in their study; especially the TW cattle. However, the possibility of missed allele calling between the two studies during merging of the datasets may have hindered the main purpose of the present study. Hence, in order to sustainably utilize the indigenous cattle in Sri Lanka, attention should be paid to genetic characterization of all the available indigenous cattle in Sri Lanka by using SNP panels before diminishing the important distinctive genetic characteristics of the indigenous cattle population of Sri Lanka.



Source: Author's own elaboration.

Figure 3: Scattergram of the first three principal components among Sri Lankan indigenous cattle

4. Conclusions

Sri Lankan indigenous cattle carry a high allelic diversity, a low genetic differentiation among geographically separated populations and a high within-population variation. Pack animals form a phenotypically separate group, although genetically they could not be distinguished from the Th cattle in this study. Time effect may have contributed to the change in the genetic structure of different cattle populations in Sri Lanka, since the Th cattle population of current study is well separated from the phenotypically similar Th1 cattle from the previous study. Findings of the current study contribute to the knowledge on genetic diversity and breeding history of Sri Lankan indigenous cattle populations.

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Molecular diversity analysis of South Indian cattle breeds

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Abstract

The present study was undertaken with the objective of evaluating the relationship between the South Indian zebu cattle populations and to identify level of genetic diversity. A total of 542 cattle from ten indigenous breeds were genotyped at 27 FAO/ISAG recommended microsatellite marker loci. All indigenous breeds studied depicted medium (0.637) to high (0.727) genetic diversity but there was inbreeding within population that might be due to consanguineous breeding practices and small effective population size. The diversity in terms of heterozygosity is moderately high in all the breeds analysed. Absence of discrete population structure observed among the breeds can be attributed to overlapping breeding or grazing tract. The genetic relatedness revealed between Hallikar and Alambadi is indeed the reason why microsatellite remains a potential tool for genetic characterization of breeds. More than 60 per cent of exotic inheritance observed in the crossbreds might predispose the animals for lower disease resistance, heat tolerance and reproductive performances. There was no bottleneck present in any of the breeds despite of the declining population size.

Keywords

zebu cattle, microsatellite, heterozygosity, diversity, admixture

1. Introduction

India has 190.9 million total cattle out of which 151 million are indigenous cattle (Ministry of Agriculture, 2012). The dairy type and dual-purpose cattle breeds are located in north and northwest India, while most indigenous draught type cattle breeds are located in southern and eastern India. After the India's "White Revolution" (a national programme in the 1970s to increase milk production and consumption), production driven farming practices have led to the decline of draught purpose animals, which were low producers of milk and gradually became less needed for ploughing, carting and transport. These breeds also faced decreased interest from farmers who found profit in rearing the high producing crossbred animals that yielded more than double the milk of indigenous draught breeds (Pundir *et al.,* 2009).

Investigation of molecular genetic diversity is a valuable complement to evaluating phenotypes and production systems in breed characterization. Microsatellites,

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also known as simple sequence repeats (SSR) or short tandem repeats (STR), are tandem repeats of one to six nucleotides that are abundantly distributed in the non-coding regions of the DNA and undergo a high rate of mutation (Field and Wills, 1996). The co-dominant nature of microsatellites made them more attractive than other genetic markers. This nature can be used to differentiate heterozygotes from homozygotes and helps to identify rare alleles. Furthermore, they are highly polymorphic and repeatable, thus providing tremendous input data to various genetic studies (Chistiakov, Hellemans and Volckaert, 2006). A comprehensive analysis including all the Indian breeds is still lacking. Hence, the present study was conducted to increase the number of DNA samples and data potentially available for a future comprehensive study, by gathering biological samples and analysing the South Indian cattle breeds at the molecular level using microsatellites. The study had two specific objectives: (i) to evaluate within breed genetic diversity of indigenous cattle populations, and (ii) to estimate genetic admixture and assess the breeds' population structure.

2. Materials and methods

A total of 542 unrelated animals representing 10 indigenous cattle populations: Deoni (n = 47), Ongole (n = 49), Punganur (n = 18), Hallikar (n = 36), Vechur (n = 26), Alambadi (n = 27), Bargur (n = 50), Kangayam (n = 50), Pulikulam (n = 34), Umblachery (n = 33), two exotic breeds: Holstein Friesian (HF) (n = 15) and Jersey (n = 34) and HF (n = 39) and Jersey crossbreds (n = 58) were collected from different regions of their native tracts. The isolated cattle genomic DNA was genotyped for 27 FAO recommended bovine specific microsatellite labelled primers (Table 1). Amplified PCR products were genotyped in an automated capillary DNA sequencer (ABI 3730 DNA Analyzer-Applied biosystems).

Table 1: Details of 27 FAO recommended microsatellite loci used to evaluate South Indian draught cattle breeds

S. No	Locus	Multiplex panel	Annealing temperature	Dye	Allele size range
1	CSRM60	1	60 °C	FAM	87-109
2	CSSM66	1	60 °C	FAM	177-197
3	HEL1	1	56 °C	HEX	96-114
4	INRA63	1	56 °C	HEX	173-183
5	BM1824	2	61 °C	ATTO550	183-197
6	ETH152	2	60 °C	FAM	189-199
7	HAUT27	2	54 °C	HEX	140-150
8	INRA05	2	54 °C	FAM	134-148
9	BM1818	3	60 °C	HEX	256-272
10	ETH3	3	63 °C	FAM	99-125
11	HEL9	3	56 °C	ATTO550	155-175
12	ILSTS006	3	54 °C	FAM	284-300
13	TGLA53	3	55 °C	HEX	153-187
14	HAUT24	4	53 °C	HEX	103-125
15	HEL5	4	54 °C	FAM	148-164
16	INRA032	4	56 °C	ATTO550	164-208
17	SPS115	4	61 °C	FAM	243-255
18	ETH185	5	65 °C	ATTO550	224-252
19	HEL13	5	54 °C	HEX	176-194
20	ILSTS05	5	56 °C	FAM	178-190
21	INRA035	5	60 °C	FAM	99-123
22	TGLA126	5	54 °C	HEX	114-128
23	BM2113	6	63 °C	FAM	118-146
24	ETH10	6	61 °C	FAM	207-225
25	ETH225	6	63 °C	ATTO550	140-162
26	INRA023	6	58 °C	ATTO550	199-219
27	TGLA122	6	58 °C	HEX	134-174

Source: Authors' own elaboration.

The allele size data for each sample was extracted and corrected in GENEMAPPER™ software. The data obtained were processed using MSA v.4.05 software for basic diversity analysis. The microsatellite markers were tested for selective neutrality using LOSITAN software (Wright, 1949) and neutral markers were selected for further analysis. A radial tree was constructed using PHYLIP v.3.5 (Felsenstein, 1993) with pair-wise Nei's genetic distances and Neighbour Joining and UPGMA algorithms. The phylogenetic tree was visualized in TREEVIEW v.1.6.6. ARLEQUIN version 3.1 (Excoffier, Laval and Schneider, 2007) was used to perform an analysis of molecular variance (AMOVA) to evaluate the distribution of microsatellite variation as a function of breed, geography and phylogeny. Multidimensional scaling of pair-wise fixation indices (F_{ST}) was undertaken along with the principal component analysis by using SPSS version 13.0. Finally, Bayesian clustering analysis was employed using STRUCTURE version 2.3.4 (Pritchard, Stephens and Donnelly, 2000), assuming $K = 2$ to $K = 15$, with a burn in period of 500 000 and a run length of 500 000 iterations.

The population was tested for mutation-drift equilibrium following the sign test, standardized differences test and Wilcoxon sign rank test under different models of microsatellite evolution as implemented in BOTTLENECK program (Piry, Luikart and Cornuet, 1999). A qualitative test of mode shift was also done to detect whether the populations had undergone any recent bottlenecks.

3. Results

Genetic diversity of breeds

A total of 13 932 genotypes were analysed over 14 breeds and 27 microsatellite markers in the study. The basic diversity statistics are given in Table 2. The mean observed number of alleles per marker per breed ranged between 9.11 (Jersey crossbreds) and 4.7 (Jersey purebreds) among all breeds, whereas it was between 8.07 (Pulikulam) and 5.93 (Punganur) among the native breeds. The mean observed heterozygosity ranged from 0.598 (Kangayam) to 0.747 (HF crossbreds) among all the breeds studied.

Hallikar showed the highest mean observed heterozygosity (0.687) among the indigenous breeds. The mean expected heterozygosity ranged between 0.63 (Jersey) and 0.77 (Jersey crossbreds) among all the breeds, and from 0.637 (Kangayam) to 0.727 (Pulikulam) among the indigenous breeds. The diversity in terms of heterozygosity was moderately high in all the breeds analysed.

Table 2: Diversity statistics for draught type zebu, taurine and crossbred cattle of South India

State of origin	Breed name	Breed code	N	n_a	H_o	H_e	F_{IS}	HWE (H_e Deficit)	HWE (H_e excess)
AP	Deoni	DEO	47	7.48	0.65	0.70	0.06	7	2
AP	Ongole	ONG	49	6.67	0.61	0.66	0.07	6	0
AP	Punganur	PNR	18	5.93	0.67	0.69	0.04	2	0
Karnataka	Hallikar	HAL	36	7.11	0.68	0.70	0.02	5	1
Kerala	Vechur	VEC	26	6.15	0.61	0.67	0.08	4	0
TN	Alambadi	ALA	27	6.48	0.67	0.70	0.05	3	1
TN	Bargur	BAR	50	6.96	0.63	0.69	0.07	7	1
TN	Kangayam	KAN	50	6.19	0.59	0.63	0.06	7	2
TN	Pulikulam	PUL	34	8.07	0.65	0.72	0.10	8	0
TN	Umblachery	UMB	33	7.63	0.64	0.71	0.08	6	0
TN	HF	HFP	15	5.81	0.64	0.70	0.09	3	1
TN	Jersey	JER	34	4.70	0.61	0.63	0.01	2	1
TN	HF CB	HFX	39	8.37	0.74	0.76	0.02	1	1
TN	Jersey CB	JRX	58	9.11	0.73	0.77	0.04	8	2

Source: Authors' own elaboration.

Hardy-Weinberg equilibrium

Out of 378 loci tested for Hardy-Weinberg Equilibrium (HWE), 69 loci over 14 breeds deviated from HWE due to heterozygosity deficit and 12 loci due to heterozygosity excess. The proportion of loci deviating from HWE was lower compared to earlier studies. For example, in Pulikulam, 12 out of 18 loci deviated from HWE in a previous study (Barani *et al.*, 2015), compared to 8 out of 27 loci in the present study.

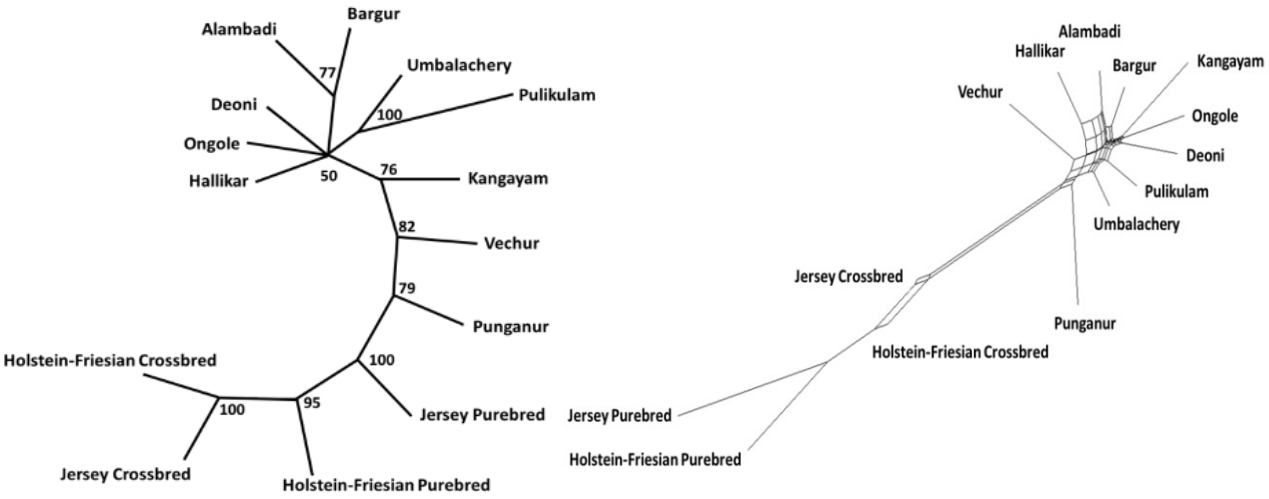
The inbreeding coefficient (F_{IS}) was positive for all the breeds analysed indicating significant heterozygosity deficiency. The mean F_{IS} value was highest in Pulikulam (0.108) and lowest in Jersey purebreds (0.017) when all breeds were considered. When the exotic breeds and

crossbreds were excluded, the breed with lowest F_{IS} was Hallikar (0.027). The mean global F_{IT}, F_{ST} and F_{IS} among zebu, exotic and crossbred cattle were 0.143, 0.101 and 0.047, respectively. The same measures in the zebu cattle were 0.109, 0.057 and 0.056, respectively. The global F_{ST} ranged from 0.052 (INRA05) to 0.223 (ETH152) among all the 14 breeds, whereas the range was from 0.024 (INRA05) and 0.114 (HAUT24) in the zebu cattle breeds.

Genetic relationship between breeds

The pairwise F_{ST} and Nei's genetic distance were calculated for all possible pairs of zebu, taurine and crossbred cattle of South India. The most closely related breeds as per the pair-wise genetic differentiation value was HF purebreds and HF crossbreds (F_{ST} = 0.007) whereas Kangayam and Jersey (F_{ST} = 0.262) were the most distantly related breeds. Among the indigenous breeds, the lowest differentiation was seen between Hallikar and Alambadi (pair-wise F_{ST} = 0.023) and it was highest between Punganur and Kangayam (F_{ST} =0.107). In accordance with the Nei's genetic distance data, HF crossbreds and Jersey crossbreds (0.0621) were the most related populations, whereas Jersey purebreds and the Ongole breed (0.5344) were the least related.

The relationship among breeds was further elucidated using phylogenetic trees, principal component analysis and multidimensional scaling. The radial tree following application of the UPGMA algorithm and the NeighbourNet tree (Figure 1) constructed based on the pair-wise Nei's genetic distances revealed groupings of indigenous cattle, purebred exotic breeds and crossbreds of South India into three distinct clusters with a 100 per cent bootstrap value (Figure 1a and Figure 1b). This supports the separate evolutionary history of the indicine and taurine populations (Loftus *et al.,* 1994). Bootstrap values suggested high robustness except for some of the topology of branches. The phylogenetic proximity of Hallikar, Alambadi and Bargur was confirmed from the genetic tree constructed based on the pair-wise Nei's genetic distance (Figure 1b). Apparently, Ongole and Deoni were closely related to each other than to other breeds in the present study.



Source: Author's own elaboration.

Figure 1: (a) UPGMA (left) and (b) NeighborNet (right) tree derived from pairwise Nei's genetic distance of zebu cattle breeds (numbers at nodes indicate percent bootstrap values of 10 000 resampled data sets)

The first three principal components derived from the inter-individual allele sharing distances were used to develop a three-dimensional scattergram using SPSS 13.0 (Figure 2). The scattergram revealed clustering of exotic and crossbreds together and separation from the indigenous breed cluster. Among the indigenous breeds, some animals of the Kangayam breed showed separation from the zebu cattle cluster. The clustering pattern was in harmony with the geographic location and evolutionary history. Multidimensional scaling of taurine, crossbreds and indigenous breeds is confounding with the PCA observations. A substructuring was observed, as Kangayam, Punganur and Vechur breeds were separated from the cluster of remaining seven breeds.

The multidimensional scaling plot (Figure 3) derived from pairwise F_{ST} values developed including zebu, crossbreds and exotic breeds revealed separate clustering of the zebu cattle and exotic cattle. The S-stress value was 0.01202 (Figure 3a). The multidimensional scaling plot of the zebu cattle alone revealed clustering of the Tamil Nadu cattle breeds with Deoni breed of Karnataka except Kangayam. Ongole and Hallikar showed a slight separation from the cluster whereas the dwarf breeds Vechur and Punganur was totally clustered separately. The S-stress value observed was 0.08001(Figure 3b).

The AMOVA revealed a high between-group variation when the grouping was done based on phylogeny, with Hallikar and Alambadi in one group; Pulikulam,

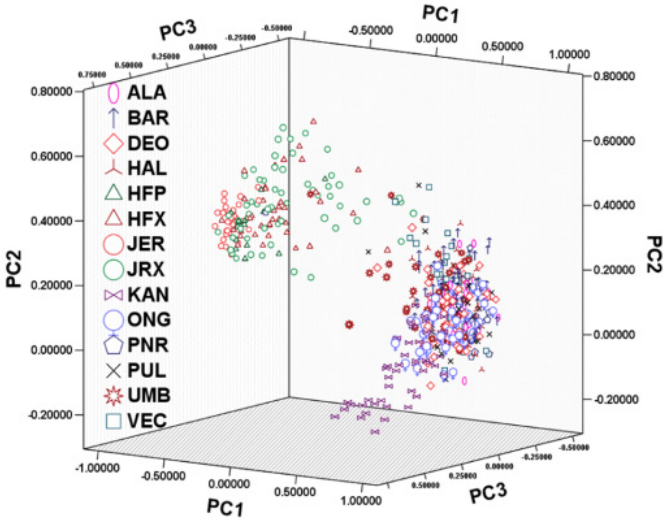
Umblachery and Bargur in a second group, Deoni and Ongole in another group; and Vechur, Punganur and Kangayam individually in separate groups. A similar grouping based on phylogeny was also found to be the best fit in the case of Asian goats (Periasamy *et al.*, 2017). The dichotomous evolution of taurine and indicine populations was reflected in the results from phylogenetic tree, principal component analysis, MDS and analysis of molecular variance.

Population structure and admixture

The population structure and level of admixture was estimated following Bayesian clustering without *a priori* knowledge of the ancestors. The Delta *K* versus *K* graph showed a peak at $K = 2$ (*Figure 4*), which represented the optimum *K* value for the investigated population based on the second order rate of change of likelihood function with respect to $K(\Delta K)$. HF and Jersey breeds were assigned to the first cluster and all the indigenous breeds to the second cluster.

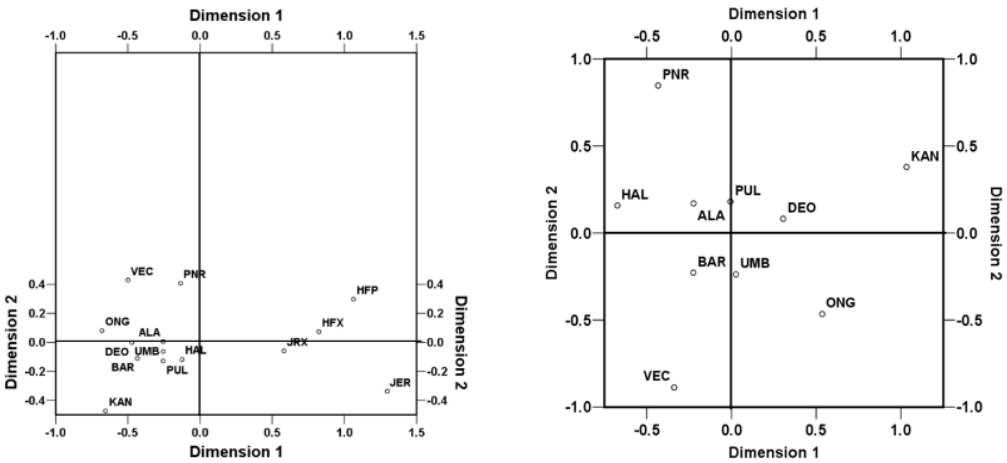
As expected, crossbreds showed admixture from both clusters. The level of exotic blood was beyond the permissible range (62.5 percent) in some of the HF and Jersey crossbred animals.

The present study also revealed that the Pulikulam, Umblachery and Alambadi breeds showed high level of admixture with other South Indian breeds, despite sampling animals with morphological features that were characteristic for each respective breed. The Kangayam, Hallikar, Deoni and Ongole were comparatively pure to their type. Ongole as a breed with minimum taurine influence (Kumar *et al.*, 2003). The availability of purebred semen for artificial insemination was seemingly a driver for maintaining the purity of these breeds.



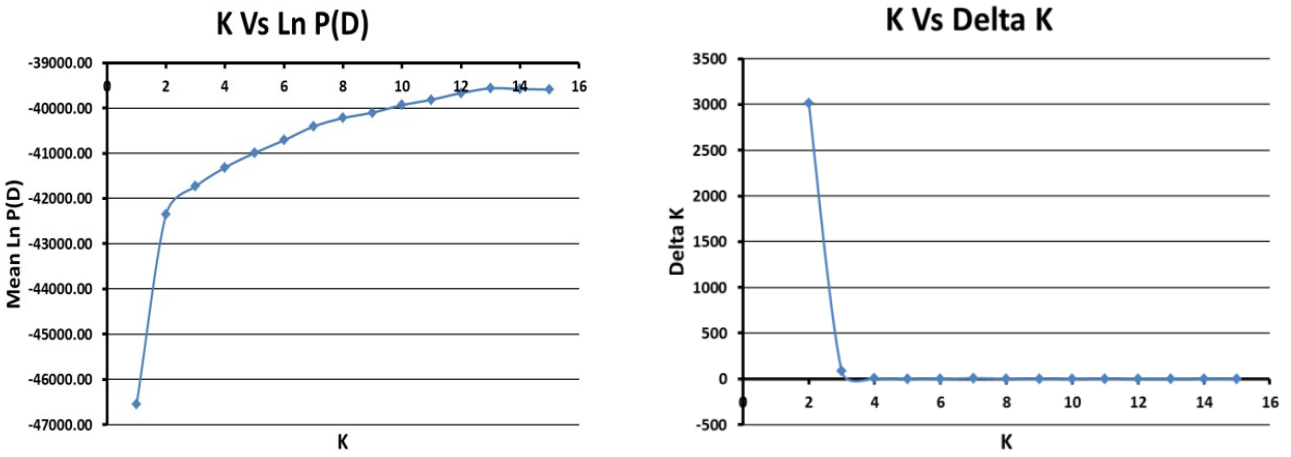
Source: Author's own elaboration.

Figure 2: Scattergram of first three largest principal components derived from pairwise inter-individual allele sharing distance among draught type zebu, taurine and crossbred cattle



Source: Author's own elaboration.

Figure 3: Multidimensional scaling display of pairwise F_{ST} among South Indian cattle: (a) draught type zebu, taurine and crossbred cattle (S-stress = 0.01202); (b) among draught type zebu cattle only (S-stress = 0.08001)



Source: Author's own elaboration.

Figure 4: Determination of correct number of clusters in Bayesian STRUCTURE analysis: (a) Mean L(*K*) over 10 runs for each *K* value of 1 to 15; (b) Distribution of Δ*K* with the modal value (*K* = 2) indicating the true *K* or the uppermost level of structure

4. Conclusions

The present study revealed moderate levels of genetic diversity existing in South Indian zebu cattle. There was no bottleneck present in any of the breeds despite of the declining population sizes. These declining population sizes could, however, lead to problems with breed sustainability in the future. To support breed conservation, parental stocks of these breeds are being established by procuring true to type animals from small holder farmers.

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Production methods, phenotype characteristics and the potential of Blackbelly Sheep in the Central Africa Forest Zone

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Abstract

This study was carried out from 1 April to 31 November 2019 on 299 Blackbelly sheep breeders in three countries, Cameroon, Congo and Gabon, to assess the exploitation methods and biodiversity. A total of 288 sheep (204 females and 84 males) were scored for 22 body measurements and 12 body indices were calculated for phenotypic diversity. This study aimed to assess the exploitation methods, phenotype characteristics and the potential of Blackbelly sheep in the Central Africa Forest Zone. Significant phenotypic dimorphism has been established between Blackbelly sheep of different countries, with the highest and heaviest animals coming from Congo (body weight = 27.44 ± 6.08 kg), longest from Gabon (body length = 84.69 ± 8.70) and the generally largest from Cameroon. A majority of the indices were larger in Cameroon sheep. The coefficients of variation of most indices were also greater in Cameroon. Principle component analysis revealed six main components among the 21 measurements of our study, which explain 73.1 percent of the variation observed. The first two components had eigenvalues greater than 3 and explained 25.44 percent and 17.41 percent, respectively, of the variation observed in body measurements and can be considered for improvement and selection programmes. Hence, back height and the thoracic circumference appear to be the most interesting measures to be considered for selection and conservation. Three classes of Blackbelly were identified in Central Africa. Class 1 includes small animals with an elongated neck. Class 2 includes the longest animals (mean body length = 86.10 cm) with a longer and larger head, body and trunk than the other classes. The ears and tail are also long, and the nipples are more developed. Class 3 includes large sheep with a voluminous chest (mean chest width = 15.12 cm) and heavy weight (mean body weight = 33.05 kg).

Keywords

exploitation, measurements, Blackbelly, biodiversity, Central Africa

1. Introduction

Sheep production is an integral part of the activities of many African people. Sheep farming is practiced for various reasons, but the most important one remains the economic profitability, which is generally accompanied by self-consumption (Tchouamo, Tchoumboué and Thibault, 2005). Blackbelly sheep have been one of the most widely distributed breeds in the world because of their high adaptive capacities in various climatic conditions (Meka zibi II *et al.*, 2019). The farming practices of Blackbelly keepers in the forest zone of Central Africa have enabled this resource to be conserved for generations, but current

environmental changes are causing changes in farming practices. No animal resource can be efficiently exploited without a qualitative and quantitative genetic inventory. These inventories include the collection of information on each breed to assess the differences between breeds and to detect, if possible, the genetic origin of these differences (FAO, 2008). To this end, several indicators of genetic diversity are commonly used, namely: breed inventories, inbreeding measures, and genetic markers (Ollivier and Foulley, 2013). As pointed out by Delgado *et al.* (2001), variations of morphological characteristics are the basis for the characterization of animal genetic resources. However, the laws of population genetics are only applicable in defining a primary breed within a context of random mating, a characteristic widely found in sub-Saharan Africa (Lauvergne *et al.,* 2011). The presence of the Blackbelly around the world demonstrates the importance and the interest given to this zoogenetic resource by economically developed countries (Meka zibi II *et al.,* 2019). Alas, in its native area, Central Africa, very little information is available about this breed and its exploitative methods. Hence, the aim of this study was to improve the knowledge of Central African small ruminant resources by assessing its exploitation methods and biometrics characteristics in its native area.

2. Materials and methods

Study area

The current study was carried out in three countries of the Central African sub-region: Cameroon, Congo and Gabon. These countries fall within the Sudano-Sahelian, Bimodal and Monomodal forest agroecological zones. For each of these countries, different administrative regions were sampled as study zones. In Cameroon, we had the South, the Littoral, the East and the Central regions. In Gabon, two regions were studied, the Wouleu-Ntem and Estuary regions. Meanwhile, in Congo, the Kouilou and Nairi regions constituted the zones of study. The bimodal rainforest agroecological zones of these countries are characterized by four seasons, two rainy seasons and two dry seasons. while the monomodal zones are characterized by single rainy dry seasons.

Measurement and data collection

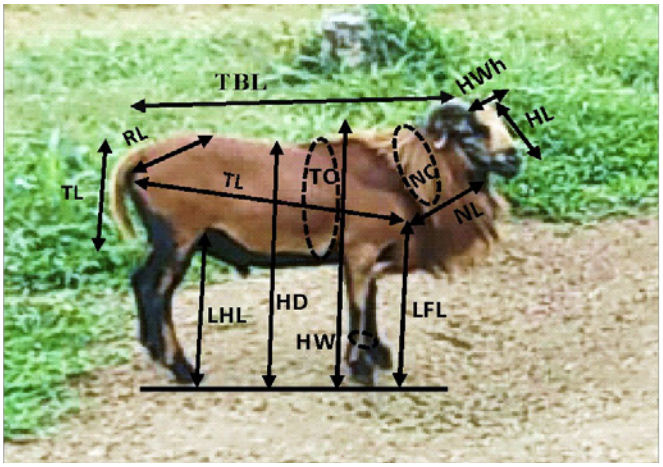
This study was based on biometric measurements collected from 288 Blackbelly sheep. A total sample of 204 females and 84 males reared in different production systems were randomly selected. To prevent any form of bias in measurements, the animals were subjected to screening and crossbreds, pregnant and unhealthy animals were eliminated from the sample. The age of the animals was determined by examination of dentition as well as direct interviews with the owners.

A total of 22 measurements were collected on each animal according to the FAO (2013) and AU-IBAR (2014) guidelines as illustrated on Figure 1.

Cephalic measurements: head length (HL), ear length (EL), head width (HWh), horn length (HLh). Body measurements: height at withers (HW), height at the back (HB), height at the rump (HR), total body length (TBL), neck length (NL), trunk length (TrL), thoracic circumference (TC), neck circumference (CN), chest depth (CD), chest width (CW), rump width (RW), rump length (RL).

Measurements of the Limbs and Extremities: length of the front leg (LFL), length of the hind leg (LHL), tail length (TL), canon bone circumference (CB), length of the nipple (LN), body weight (BW).

Body indices were determined from the 22 measurements following the methodologies of Arredondo-Ruiz *et al.* (2013) and Ngono, Meutchieyé and Manjeli (2019) to appreciate the functional relationship that exists between the measurements. Subsequently, the following 12 indices were calculated: substernal gracility index (SGI), ear length



Source: Author's own elaboration.

Figure 1: Some of the body measurements of Blackbelly in this study

index (ELI), format index (IF), compactness index (CI), massiveness index (MI), chest index (ChI), slenderness index (SI), frame index (FI), body index (BI), dactylo-thoracic index (DTI), caudal index (ICa), ear index (EI).

Statistical analyses

Descriptive statistics were used and calculated for the different measurements as well as the indices. Analysis of variance was used to test the influence of country on body measurements while the *t*-test was used to evaluate the influence of sex on measurements and indices.

The following statistical model was adopted: $Y_{ik} = \mu + a_i + e_{ik}$, where: Y_{ik} is the performance (measurement) of the *k*th animal of country *I*; μ is the overall population mean; a_i is the effect of the country *i (i varying from 1 to 3)*; e_{ik} is the residual error of the *k*th individual of country *i*.

Duncan's Multiple Range Test was used to separate the means when the effects of the country and the sex were significant ($p \leq 0.05$). The 22 biometric measurements were subjected to principal component analysis (PCA) to determine the linear relationship that exists among these characteristics (FAO, 2013). The overall adequacy of the PCA was established by the Kaiser-Meyer-Olkin (KMO) test of sampling and Bartlett's test of sphericity (Kaiser, 1960; Eyduran *et al.*, 2010).

Based on the 22 body measurements, discriminant factor analysis (DFA) was used to identify the morphological types found within the studied population (Faye *et al.*, 2011; FAO, 2013) in a bid to assess and or validate the homogeneity of this breed. The relationship that existed between the different morphological groups was established through ascendance hierarchical clustering (AHC) (Carpentier, 2007).

Statistical analyses were carried out with SPSS 21 (2018) and R Core Team (2018).

3. Results and discussion

Biometric characteristics of Blackbelly sheep in Central Africa

Descriptive analysis of biometric measurements

Table 1, Table 2 and Table 3 present the descriptive statistics of the body measurements according to the countries studied. With respect to cephalic measurements (Table 1) the highest HWh, NL and EL were recorded for the Blackbelly population of Congo, meanwhile those in Gabon presented a higher HLh and CN. Only the EL and CN differed ($p \leq 0.05$) among countries. Ear Length was significantly higher in the Blackbelly population of Congo (11.23 ± 1.16 cm) when compared to those in Gabon (9.09 ± 1.80 cm), which presents a large dispersion value (18.96 percent). A statistically higher CN (34.355 ± 8.324 cm) was obtained for the Blackbelly population of Gabon with a 23.10 percent coefficient of variation when compared to that of Congo. Table 2 shows that, except for HWh, TC and RW, every other body measurement differed ($p \leq 0.05$) between at least two countries. The greatest average TBL (84.691 ± 8.704 cm) and TL (62.57 ± 5.88 cm) were recorded for the Gabon population, meanwhile the largest HWh, HB, TC and CD were observed for those in Congo. The average CW, RL and RW were highest in Cameroon. With respect to the measurements of the limbs, extremities and body weights (Table 3), significant ($p \leq 0.05$) differences were observed only for LFL, TL and CB. The average LFL, LHL, CB and LN were highest in the Blackbelly sheep population of Cameroon, but the highest BW was recorded for Congo.

Descriptive analysis of biometrics indices

Except for the MI, the DTI and the ICa, results (Table 4 and Table 5) reveal a significant difference ($p \leq 0.05$) in the biometric indices (SGI, ELI, IF, CI, IT, SI, FI, BI, EI) of the Blackbelly sheep population in the different countries studied. The highest value for most biometric indices (SGI, ELI, CI, MI, IT, SI, FI, DTI, EI) were obtained for the Cameroon Blackbelly sheep population as well as their corresponding coefficients of variations (except for IT and FI).

Correlations between body measurements in Blackbelly sheep from Central Africa

Several significant correlations were observed between the biometric measurements of the Blackbelly sheep population of Central Africa (no table shown). The coefficients ranged from -0.297 (i.e. between NL and the HB) to 1.00 (i.e. TC and BW). A very strong correlation (0.94) was observed between the HW and HB. Body weight was also significantly ($p < 0.01$) and positively correlated with HW (0.72), HB (0.70) and HR (0.66).

Variability of body measurements

Table six shows the contributions of the various body measurements to the first six principal components. These components explained 73.1 percent of the total. The Kaiser-Meyer-Olkin (KMO) index for the efficiency of the sample for PCA of biometric measurements was 0.741. The test for the total significance of the correlation between body measurements ($X^2 = 4251.603$ $p < 0.01$) as well as the sphericity test of Bartlett were significant, thus further supporting the validity of the factor analysis for our data. The six principal components in Table 6 had eigenvalues greater than 1. Components 1 and 2 with eigenvalues of 5.598 and 3.835, respectively, cumulatively they represented 42.87 percent of the phenotypic variability observed

As shown in Table 6, HW, HB, HR, TC and BW contribute substantially to the total variability observed in component 1. The RL, LFL and LHL best explain and positively relate to the variability in the second component.

Table 1: Head width, head length, horn length, neck length, circumference of neck and ear length of Blackbelly sheep in Central Africa

	Country	*n*	Mean ± SD	CV (%)
HWh	Cameroon	252	10.829 [a] ± 1.466	13.51
	Congo	24	11.450 [a] ± 0.926	7.81
	Gabon	11	10.791 [a] ± 1.158	10.23
	Average	**287**	**10.880 ± 1.425**	**13.05**
HL	Cameroon	252	18.756 [a] ± 9.962	53.1
	Congo	24	17. 621 [a] ± 1.736	9.56
	Gabon	11	16.664 [a] ± 1.218	6.97
	Average	**287**	**18.581 ± 9.361**	**50.37**
HLh	Cameroon	252	4.405 [a] ± 7.5545	171.17
	Congo	24	4.058 [a] ± 8.100	178.73
	Gabon	11	8.009 [a] ± 10.732	127.76
	Average	**287**	**4.514 ± 7.7387**	**171.39**
NL	Cameroon	252	25.167 [a] ± 6.892	27.33
	Congo	24	26.867 [a] ± 6.111	22.58
	Gabon	11	26.527 [a] ± 2.355	8.47
	Average	**287**	**25.361 ± 6.720**	**26.49**
CN	Cameroon	252	32.282 [ab] ± 4.574	14.14
	Congo	24	30.488 [a] ± 6.018	18.95
	Gabon	11	34.355 [b] ± 8.324	23.10
	Average	**287**	**32.211 ± 4.9120**	**15.43**
EL	Cameroon	252	9.657 [ab] ± 1.550	16.02
	Congo	24	11.233 [b] ± 1.165	10.36
	Gabon	11	9.091 [a] ± 1.807	18.96
	Average	**287**	**9.767 ± 1.594**	**16.29**

Source: Authors' own elaboration.

Notes: a [a, b] the means assigned the same subscript in the same column indicate that there are no significant differences between countries ($p > 0.05$).

b **HL** = head length, **HWh** = head width, **HLh** = horns length, **EL** = ear length, **NL** = neck length, **CN** = neck circumference.

Table 2: Length of body and trunk, height at withers, back and rump, circumference, depth, width of the chest and length, width of the rump of the Blackbelly in Central Africa

	Country	*n*	Mean ± SD	CV (%)
TBL	Cameroon	252	78.563 [a] ± 8.982	11.41
	Congo	24	83.321 [ab] ± 7.384	8.50
	Gabon	11	84.691 [b] ± 8.704	9.80
	Average	**287**	**79.196 ± 8.988**	**11.34**
TRL	Cameroon	252	52.421 [a] ± 10.280	19.57
	Congo	24	60.000 [b] ± 7.431	11.91
	Gabon	11	62.573 [b] ± 5.885	8.97
	Average	**287**	**53.444 ± 10.302**	**19.27**
HW	Cameroon	252	59.315 [a] ± 7.142	12.02
	Congo	24	62.217 [a] ± 5.288	8.18
	Gabon	11	57.855 [a] ± 6.288	10.36
	Average	**287**	**59.502 ± 7.011**	**11.78**
HB	Cameroon	252	58.075 [ab] ± 7.158	12.30
	Congo	24	59.229 [b] ± 5.221	8.45
	Gabon	11	54.418 [a] ± 4.992	8.75
	Average	**287**	**58.032 ± 6.976**	**11.88**
HR	Cameroon	252	58.897 [b] ± 6.580	11.15
	Congo	24	58.383 [b] ± 4.720	**7.77**
	Gabon	11	53.727 [a] ± 3.983	**7.07**
	Average	**287**	**58.656 ± 6.429**	**10.94**
TC	Cameroon	252	71.508 [a] ± 9.023	12.59
	Congo	24	72.096 [a] ± 6.864	9.30
	Gabon	11	69.155 [a] ± 8.397	11.58
	Average	**287**	**71.467 ± 8.829**	**12.34**
CD	Cameroon	252	25.919 [a] ± 4.845	18.66
	Congo	24	36.713 [b] ± 3.455	9.50
	Gabon	11	34.200 [b] ± 3.806	10.61
	Average	**287**	**27.139 ± 5.744**	**21.15**
CW	Cameroon	252	15.016 [b] ± 2.531	**16.82**
	Congo	24	11.804 [a] ± 2.544	**20.75**
	Gabon	11	12.436 [a] ± 2.045	**15.68**
	Average	**287**	**14.648 ± 2.697**	**18.41**
RL	Cameroon	252	20.331 [b] ± 6.480	31.81
	Congo	24	17.517 [ab] ± 2.409	13.21
	Gabon	11	16.164 [a] ± 2.522	14.88
	Average	**287**	**19.936 ± 6.223**	**31.19**
RW	Cameroon	252	14.716 [a] ± 2.351	15.95
	Congo	24	14.233 [a] ± 1.876	12.73
	Gabon	11	14.155 [a] ± 2.582	17.40
	Average	**287**	**14.654 ± 2.323**	**15.83**

Source: Authors' own elaboration.

Notes: a [a, b] the means assigned the same letter in the same column indicate that there are no significant differences between countries ($p > 0.05$).

b **TBL** = total body length, **TrL** = trunk length, **HW** = height at withers, **HB** = height at back, **HR** = height at rump, **TC** = thoracic circumference, **CD** = depth of chest, **CW** = width of chest, **RW** = Rump width, **RL** = Rump length.

Table 3: Length of the front leg, length of the hind leg, tail length, canon bone circumferences, length of the nipple, body weight of the Blackbelly in Central Africa

	Country	*n*	Mean ± SD	CV (%)
LFL	Cameroon	252	42.885 [b] ± 7.405	17.23
	Congo	24	37.733 [ab] ± 4.863	12.38
	Gabon	11	35.709 [a] ± 2.587	6.91
	Average	**287**	**42.179 ± 7.346**	**17.40**
LHL	Cameroon	252	45.008 [a] ± 7.352	16.30
	Congo	24	44.663 [a] ± 3.988	8.65
	Gabon	11	42.255 [a] ± 2.504	5.65
	Average	**287**	**44.874 ± 7.015**	**15.62**
TL	Cameroon	252	22.448 [ab] ± 5.729	25.47
	Congo	24	24.871 [b] ± 4.017	15.48
	Gabon	11	21.100 [a] ± 4.021	18.17
	Average	**287**	**22.599 ± 5.586**	**24.70**
CB	Cameroon	252	7.823 [b] ± 0.798	10.18
	Congo	24	7.254 [a] ± 0.747	9.92
	Gabon	11	7.164 [b] ± 0.815	10.85
	Average	**287**	**7.751 ± 0.816**	**10.45**
LN	Cameroon	252	1.637 [a] ± 1.369	83.48
	Congo	24	1.538 [a] ± 1.435	95.49
	Gabon	11	1.145 [a] ± 1.121	93.31
	Average	**287**	**1.610 ± 1.365**	**84.47**
BW	Cameroon	252	26.926 [a] ± 7.994	29.63
	Congo	24	27.447 [a] ± 6.081	21.74
	Gabon	11	24.841 [a] ± 7.439	28.56
	Average	**287**	**26.890 ± 7.822**	**29.08**

Source: Authors' own elaboration.

Notes: a [a, b] the means assigned the same letter in the same column indicate that there are no significant differences between countries ($p > 0.05$).

b **LFL** = length of the front leg, **LHL** = length of the hind leg, **TL** = tail length **CB** = canon bone circumferences, **LN** = length of the nipple, **BW** = body weight.

Table 4: Variation of sub-sternal gracility index, ear length index, format index, compactness index, massiveness index, thoracic index of Blackbelly per country in Central Africa

Index	Country	*n*	Mean ± SD	CV (%)
SGI	Cameroon	252	1.375 [b] ± 0.5476	39.75
	Congo	24	0.699 [a] ± 0.0999	14
	Gabon	11	0.702 [a] ± 0.1893	25.70
	Total	**287**	**1.293 ± 0.5605**	**43.41**
ELI	Cameroon	252	0.389 [b] ± 0.1133	28.56
	Congo	24	0.307 [a] ± 0.0315	9.84
	Gabon	11	0.269 [a] ± 0.0657	23.28
	Total	**287**	**0.377 ± 0.1099**	**27.02**
IF	Cameroon	252	1.334 [a] ± 0.1553	11.62
	Congo	24	1.345 [a] ± 0.1316	9.39
	Gabon	11	1.472 [b] ± 0.1613	10.45
	Total	**287**	**1.340 ± 0.1555**	**11.19**
CI	Cameroon	252	0.914 [b] ± 0.1014	11.07
	Congo	24	0.867 [ab] ± 0.0648	7.39
	Gabon	11	0.819 [a] ± 0.0901	10.48
	Total	**287**	**0.907 ± 0.1006**	**11.11**
MI	Cameroon	252	1.209 [a] ± 0.1255	9.29
	Congo	24	1.160 [a] ± 0.0807	6.78
	Gabon	11	1.198 [a] ± 0.1168	9.29
	Total	**287**	**1.205 ± 0.1109**	**9.16**
ChI	Cameroon	252	0.213 [b] ± 0.0402	18.87
	Congo	24	0.166 [a] ± 0.0479	27.61
	Gabon	11	0.180 [a] ± 0.0238	12.57
	Total	**287**	**0.207 ± 0.0426**	**19.32**

Source: Authors' own elaboration.

Notes: a [a, b] the indices affected by the same letter in the same column indicate that there are no significant differences between the countries ($p > 0.05$).
b **SGI** = substernal gracility index, **ELI** = ear length index, **IF** = format index, **CI** = compactness index, **MI** = massiveness index, **ChI** = chest index.

Table 5: Variation of slenderness index, frame index, body index, dactylo-thoracic index, caudal index, ear index of Blackbelly sheep in Central Africa

Index	Country	*n*	Mean ± SD	CV (%)
SI	Cameroon	252	0.557 [b] ± 0.0970	17.38
	Congo	24	0.410 [a] ± 0.0329	8.34
	Gabon	11	0.407 [a] ± 0.0582	13.65
	Average	**287**	**0.539 ± 0.1041**	**18.86**
FI	Cameroon	252	0.133 [b] ± 0.0188	14.03
	Congo	24	0.117 [a] ± 0.0104	8.53
	Gabon	11	0.125 [ab] ± 0.0191	14.57
	Average	**287**	**0.132 ± 0.0188**	**7.69**
BI	Cameroon	252	0.747 [a] ± 0.1736	23.20
	Congo	24	0.833 [ab] ± 0.0709	8.21
	Gabon	11	0.911 [b] ± 0.0819	8.58
	Average	**287**	**0.760 ± 0.1690**	**21.05**
DTI	Cameroon	252	0.111 [a] ± 0.0172	15.46
	Congo	24	0.101 [a] ± 0.0101	9.62
	Gabon	11	0.104 [a] ± 0.0115	10.47
	Average	**287**	**0.110 ± 0.0168**	**14.54**
Ica	Cameroon	252	0.381 [a] ± 0.0915	23.99
	Congo	24	0.400 [a] ± 0.0557	13.377
	Gabon	11	0.364 [a] ± 0.0496	13.00
	Average	**287**	**0.382 ± 0.0879**	**20.94**
EI	Cameroon	252	0.164 [ab] ± 0.0274	16.67
	Congo	24	0.181 [b] ± 0.0156	8.59
	Gabon	11	0.157 [a] ± 0.0305	18.51
	Average	**287**	**0.165 ± 0.0271**	**12.12**

Source: Authors' own elaboration.

Notes: a [a, b] the indices affected by the same letter in the same column indicate that there are no significant differences between the countries ($p < 0.05$).

b **SI** = slenderness index, **FI** = frame index, **BI** = body index, **DTI** = dactylo-thoracic index, **ICa** = caudal index, **EI** = ear index.

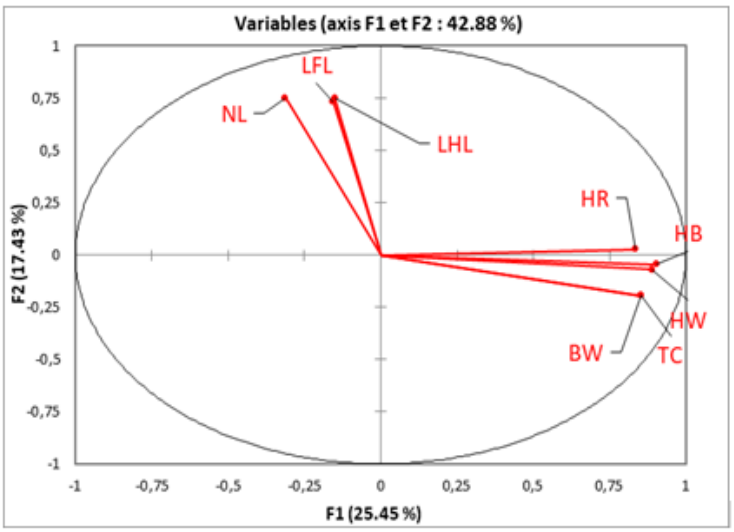
Table 6: Matrix of components for the different body measurements of Blackbelly sheep from Central Africa

Variable	Component					
	1	2	3	4	5	6
HWh (cm)	0.534	0.307	-0.425	0.172	0.011	-0.046
HL (cm)	0.166	0.126	-0.002	0.172	0.128	0.156
HLh (cm)	0.206	-0.043	-0.416	-0.626	0.374	-0.176
NL (cm)	-0.313	**0.752**	0.332	0.093	0.005	-0.138
CN (cm)	0.543	0.411	-0.315	-0.300	0.230	0.061
EL (cm)	0.386	0.067	-0.160	0.114	-0.479	-0.389
TBL (cm)	0.675	-0.118	-0.113	0.330	0.262	-0.200
TrL (cm)	0.121	**0.644**	-0.413	0.445	0.053	-0.164
HW (cm)	**0.890**	-0.071	0.249	-0.152	-0.148	-0.090
HB (cm)	**0.903**	-0.044	0.082	-0.144	-0.240	0.010
HR (cm)	**0.835**	0.027	0.322	-0.186	-0.163	-0.002
TC (cm)	**0.853**	-0.196	0.209	0.052	0.111	0.166
CD (cm)	0.043	0.540	0.445	0.148	0.190	-0.530
CW (cm)	0.065	0.301	0.383	-0.012	0.536	0.449
RL (cm)	0.430	0.162	**-0.690**	0.231	-0.181	0.251
RW (cm)	0.100	**0.670**	-0.032	0.361	0.404	0.049
LFL (cm)	-0.156	**0.733**	0.024	-0.232	-0.416	0.329
LHL (cm)	-0.150	**0.751**	0.065	-0.249	-0.379	0.187
TL (cm)	0.342	0.478	0.262	-0.138	-0.083	-0.304
CB (cm)	0.277	0.501	-0.033	-0.216	0.054	0.254
LN (cm)	0.159	-0.180	0.262	**0.667**	-0.287	0.300
BW (kg)	**0.853**	-0.196	0.209	0.052	0.111	0.166

Source: Authors' own elaboration.

The projection in Figure 2 discriminates between two main groups of variables that are negatively correlated with each other (NL, LFL, LHL on the F2 axis and HW, HB, HR, BW, TC on the F1 axis)

It appears that, the HW, HB, HR, the TC and BW are positively and strongly correlated as well as with the F1 axis. However, NL, LFL and LHL are strongly correlated with each other and with the F2 axis.



Note: ***NL*** = Neck length, ***LFL*** = Length of the front leg, ***LHL*** = Length of the hind leg, ***HW*** = Height at withers, ***HB*** = Height at the back, ***HR*** = Height at the rump, ***TC*** = Thoracic circumference and ***BW*** = Body weight.
Source: Author's own elaboration.

Figure 2: Correlation circle of the initial variables by PCA for body measurements of Blackbelly sheep in Central Africa

Population Structure Based on Body Measurements

Table 7 further highlights the distinction that exists within the Blackbelly sheep population. The various traits measured in this study made it possible to determine three morphological groups and the average biometric characteristics of these types (see Table 7). The most discriminating factors that characterize the group 1 sheep are NL, LFL and LHL. The population which constitutes the second are discriminated by HWh, HL, CN, EL, TBL, TrL, HB, CD, RL, RW, CB and LN. Finally, the following biometric measurements discriminate group 3 sheep: HLh, HW, HR, TC, CW and BW. Most of the largest mean measurements were observed in the group 2.

Table 7: Biometric characteristics of the 3 morphological types of Blackbelly sheep in Central Africa

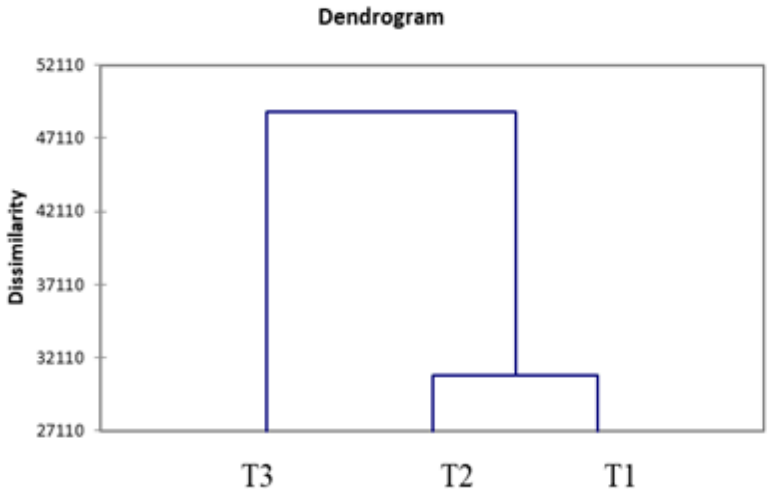
Variables	Classes		
	1	2	3
HWh (cm)	10.451	**11.937**	10.272
HL (cm)	16.941	**21.284**	18.075
HLh (cm)	3.745	5.1626	5.325
NL (cm)	**29.183**	23.743	19.924
CN (cm)	31.204	**34.427**	31.121
EL (cm)	9.3854	**10.442**	9.590
TBL (cm)	73.831	**86.108**	80.378
TrL (cm)	55.083	**61.895**	38.606
HW (cm)	54.856	62.905	**64.045**
HB (cm)	53.551	**62.234**	61.151
HR (cm)	55.458	60.485	**62.500**
TC (cm)	64.464	76.442	**78.424**
CD (cm)	27.855	**28.1123**	24.424
CW (cm)	14.904	13.89	15.121
RL (cm)	18.515	**25.491**	15.075
RW (cm)	15.190	**15.430**	12.5
LFL (cm)	**44.618**	41.914	37.666
LHL (cm)	**46.929**	45.162	40.439
TL (cm)	23.132	23.276	20.65
CB (cm)	7.783	**7.827**	7.568
LN (cm)	1.133	**2.0494**	1.924
BW (kg)	20.685	31.298	**33.053**

Source: Authors' own elaboration.

Note: **HL**: Length of the head, **HWh**: Width of the head, **HLh**: Length of the horns, **EL**: Ear length, **NL**: Neck length, **CN**: Circumference of the neck, **TBL**: Total body length, **TrL**: Trunk length, **HW**: Height at withers, **HB**: Height at back, **HR**: Height at rump, **TC**: Thoracic circumference, **CD**: Chest depth, **CW**: Chest width, **RW**: Rump width, **RL**: Rump length, **LFL**: Length of the front leg, **LHL**: Length of the hind leg, **TL**: Length of the tail, **CB**: Canon bone circumferences, **LN**: Length of the nipple, **BW**: Body weight.

Relationships between the morphological types of Blackbelly sheep populations in Central Africa as a function of body measurements

Despite clear differences among the group means shown in Table 7, the AHC suggested that the Blackbelly sheep population constituted of only two subgroups (See Figure 3). The first subgroup is composed of morphological type 3 (T3) while groups 1 and 2 (types T2 and T1) constituted the second subgroup. Therefore, T1 is more closely related to T2 but distantly related to T3. This result may possibly be due in part to the genetic distances that exist between the subgroups, although we had no pedigree or molecular data to substantiate this hypothesis. This could hint to the possibility that T1 and T2 share a common genetic heritage.



Source: Author's own elaboration.

Figure 3: Dendrogram of the Blackbelly sheep population morphotypes in Central Africa

4. Discussion

The biometric data obtained from the Central African Blackbelly population in the different countries studied revealed a great phenotypic variability ($p \leq 0.05$). This may point to the existence of a significant genetic diversity (Gaouar *et al.*, 2015). On a general note, the HWh and HLh) in this study were 18.581 ± 9.361 cm and 10.880 ± 1.425 cm, respectively. These results are comparable between countries. This result is similar to that reported by Tadakeng (2015) on the West Cameroon sheep (20.04 ± 3.34 cm). Animals of the Congo Blackbelly sheep population had significantly longer ears (11.233 ± 1.165 cm) than those in other countries. Similar results were obtained by Dayo *et al.* (2015) on Djallonke sheep in Togo (11.613 ± 2.61 cm). However, these values are low compared to those obtained by Dayo *et al.* (2015) in Togo on the Vogon (18.45 ± 2.08 cm) and Sahelian (21.631 ± 2.48 cm) breeds. These differences can be explained by the fact that, the Vogon and Sahelian breeds possess characteristic droopy or semi pendulous ears which is probably an adaptive mechanism for efficient thermoregulation in hot zones. The overall mean TBLs obtained for the different countries studied were 79.196 ± 8.98 cm. These values are higher than those obtained by Arora, Bhatia and Jain (2010) on Ganjam sheep in India (76.75 ± 12.28 cm) and Wilson (1992) on the Djallonké sheep (60–65 cm). Nevertheless, they are comparable to those obtained by Tadakeng (2015) on the West Cameroon sheep (76.75 ± 12.28 cm).

Although significantly ($p < 0.05$) higher for the Congo Blackbelly population, the overall mean TrL obtained in this study (53.44 ± 10.302 cm) is close to that reported by Tadakeng (2015) on West Cameroon sheep (54.96 ± 9.06 cm). Though variable among the countries studied (Table 2), an overall average HW of 59.502 ± 7.011 was recorded. Arora, Bhatia and Jain (2010) and Barra *et al.* (2015) reported larger values for the Ganjam (67.7 ± 0.48 cm) and Merinos (67.88 ± 3.53 cm) sheep in India and Chilie, respectively. Our result remains comparable to that obtained by Vallerand and Branckaert (1975) on the Djallonké sheep (59 cm) and Tadakeng (60.59 ± 8.30 cm) sheep breed of the Cameroon Western Highlands, which are likely to have greater similarity to ours in terms of both environmental and genetic effects than the two exotic breeds. A mean chest circumference of 71.467 ± 8.829 cm was obtained from our data, but no statistical difference ($p > 0.05$) between countries was observed. A similar value was reported by Arora, Bhatia and Jain (2010) for the Ganjam sheep (72.7 ± 0.68 cm) of India.

Results revealed a mean RL and RW of 19. 93 ± 6.22 cm and 14.64 ± 2.32 cm, respectively. A lesser mean rump width was reported by Denis (1975) for the Peul sheep of Senegal (14.4 ± 0.5 cm) and Sibomana (1998) for the local Rwandan and Burundian breeds (16.89 ± 4.68 cm). Indeed, a larger rump would appear to be a characteristic of good mothering quality in the ewe, which further confirms the observations of Manjeli *et al.* (1996) on the prolificity of the Blackbelly sheep in Cameroon. An overall mean BW of 26.89 ± 7.82 kg was observed. Traoré

et al. (2006) in Burkina Faso and Birteeb, Peters and Ozoje (2014) in northern Ghana reported lower body weights for the Mossi sheep (23.3 ± 5.0 kg) and Djallonke sheep (26.92 ± 0.89 kg), respectively. With a bodyweight lower than 35 kg, we can still conclude that the Blackbelly sheep of Central Africa are generally smaller in size relative to many other breeds.

The analysis of indices revealed a significant ($p \leq 0.05$) viability between the Blackbelly sheep population of the countries studied. The mean SGI and ELI of 1.29 ± 0.56 and 0.37 ± 0.10, respectively, suggest that the Blackbelly sheep is of intermediate type. Gueye (1997), however, reported dissimilar SGI for the following sheep breeds: 0.59 for the Djallonké sheep, 1.61 for the Touabire sheep and 1.51 for the Peuhl sheep. The high indices reported for the sheep breeds of the Sahelian zone (i.e. the latter two) suggest an adaptation for efficient thermoregulation. This is because an increase in leg length with a corresponding increase in SGI, enables the animal to reduce the thermal impact of the infrared radiation from the ground as well as the solar radiation reflected by the soil on the body (Zeuh *et al.*, 1997). The sheep from Cameroon presented a significantly higher CI (0.914) than those from Gabon. Similar results were obtained in Tadakeng (2015) on the West Cameroon sheep. The MI gives an appraisal of the relationship between the body extremities and body mass. Results reveal a mean MI of 1.20 ± 0.11 which is close to the findings of Tadakeng (2015) obtained for the West Cameroon sheep (1.13). However, authors who worked on closely related ruminant species reported dissimilar findings in the instances of Chacón *et al.* (2011) and Ngono, Meutchieyé and Manjeli (2019) who recorded MI of 0.57 for the Creole goat of Chile and 2.72 for the White Fulani cattle of Cameroon, respectively. The MI is a functional index that plays a role in the animal's adaptation to its environment. Results show that the mean ChI of 0.2 ± 0.0426 with a significantly higher value recorded in favour of the Cameroon sheep (0.213). Arredondo-Ruiz *et al.* (2013) on the other hand recorded a higher mean ChI (0.5) for the Peliluey sheep population in Mexico.

The BI enables us to have an overall appraisal of the size of the animal. Following the classification model adopted by Chacón *et al.* (2011), where, if the BI > 0.90: "long", BI = 0.86 – 0.88: "medium" and BI < 0.85: "stocky", we could conclude that the Blackbelly sheep of Central Africa is generally compact (BI = 0.760). However, the subset of Gabon presented a longer linear body conformation (0.911). According to the DTI, sheep can be classified into four categories: lightweight (DTI < 0.105); intermediary (DTI = 0.106 – 0.108); heavyweight (DTI = 0.109 – 0.11); and very heavy (0.111 < DTI) breeds. It is safe to say that the Blackbelly sheep of Central Africa is a heavyweight meat type (DTT = 0.110). Putra and Ilham (2019) obtained different values in his studies carried out on the Katjang goat of Indonesia. The reports of Arredondo-Ruiz *et al.* (2013) indicate lower values for both sexes (0.097 in male and 0.095 in female) of the Pelibuye breed in Mexico. The DTI values obtained for the Cameroon Blackbelly sheep are similar to the findings of Tadakeng (2015) who recorded a DTI of 0.12 for the West Cameroon sheep. The DTI equally provides information on the dairy capability of breeds (Álvarez *et al.*, 2009).

The correlations among traits corroborate the "theory of Bergam" on animal growth. The highly positive correlations between the biometric parameters of the Blackbelly sheep of Central Africa are similar to those reported by Arredondo-Ruiz *et al.* (2013) for the Pelibuye sheep of Mexico. This concurrence could highlight a common origin for both breeds (Meka zibi II *et al.*, 2019).

The PCA of 6 major components account for 73.1 percent of the total phenotypic variability observed, and components CP1 and CP2 explained 25.44 percent and 17.41 percent, respectively. Contrarily, divergent results have been reported by several authors. Yakubu (2013) extracted three, while Osaiyuwu, Akinyemi and Salako (2010) reported two main components after subjecting the body measurements collected from the respective Yankasa sheep and Balami sheep populations of Nigeria to PCA. In the same line, Putra and Ilham (2019) reported four main components to be responsible for most of the variability observed in the Katjang goat population of Indonesia. Nonetheless, the results of this study agree with the findings of Barra *et al.* (2015) who reported that 6 main components explained the phenotypic variability observed in the sheep population of Chile. Nevertheless, the common denominator in all these results is the fact that the first component (CP1) explained a major part of the observed phenotypic variability and thus can be used for selection programmes to obtain more stable breeds. The PCA distinguished the height variables (HW, HB and HR) plus BW and TC to explain the maximum variation in CP1, and the length and width variables (NL, TrL, RL, RW, LFL, LHL) to account for the variations in CP2. The TC was reported to have the greatest contribution to the rotating CP1 in a study carried out on the Yankassa sheep population of Nigeria (Yakubu, 2013), a result which contrasts the findings of this study, where the greatest contribution to the same rotating CP1 was from HB.

5. Conclusions

There is great phenotypic variation among the Blackbelly sheep of Central Africa. The longest animals were from Gabon while the highest, largest, and heaviest were from Congo. Relative to other breeds of sheep found across the world, Blackbelly sheep of Central Africa are small in size. The trait TC can efficiently be used as a proxy for BW, as we obtained a perfect correlation between these measurements. This biometrics characterization of the breed is only a first step. Conventional selection

and possibly the application of genomics and other biotechnologies need also to be used in order to improve the performance of the Central Africa Blackbelly sheep.

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Inventory and nutritional value of local fodder resources during the dry season in Menoua Division, Western Highland of Cameroon

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Abstract

This study was carried out in the Menoua division, western region of Cameroon from January to June 2021 to develop a data bank of local fodder resources consumed by small ruminants during the dry season. Three villages per district where small ruminant breeders were most represented were chosen. A survey was carried out to collect information on the fodder resources consumed by small ruminants. From each fodder identified, a sample was collected for proximate analysis. The results revealed that 21 fodder species were used to feed small ruminants during the dry season in the Menoua division. The most fodders species known by farmers were *Pennisetum purpureum, Desmodium uncinatum, Musa paradisiaca* and *Manihot esculenta.* Goats and sheep generally consumed the leaves and most of these forages were at the flowering stage during the dry season. The fodder species belonging to the Poaceae family had the highest fibre content ranging from 25.87 percent to 36.25 percent dry matter (DM), while those belonging to the Fabaceae family had the highest crude protein contents ranging from 25.02 percent to 36.72 percent DM. *Manihot esculenta* recorded the highest contents of Milk Forage Unit (107.83 FU kg of DM), Meat Forage Unit (101.92 FU kg of DM) and *Gliricidia sepium* recorded the highest content of digestible protein in the intestine allowed by nitrogen (23.08 g/kg DM). These results constitute the main support for the synthesis and dissemination of knowledge on the nutritional value of local fodder for feeding small ruminants during the dry season.

Keywords

dry season, nutritional value, local fodder, small ruminants, Cameroon.

1. Introduction

In Western Highland of Cameroon, small ruminant rearing is a major activity in the livelihoods of the population and contributes greatly to income generation in the region and therefore alleviating poverty. In this area,

villagers practice sedentary agriculture, but they also keep relatively large herds of livestock to augment income and meet dietary and subsistence needs (Lemoufouet, Pamo and Tendonkeng, 2012). The staple food of ruminants consists of fodder, the diversity and nutritional quality of which is a recurring problem for breeders, since it must simultaneously meet the nutritional needs for both maintenance and production of animals (Lemoufouet, Pamo and Tendonkeng, 2012; Zirmi-Zembri and Kadi, 2016).

In this area, the identification and characterization of fodder is of great importance because the zootechnical performance of livestock is strongly linked to the nutritional value of pastures (Pamo *et al.,* 2007; Ahmad, Singh and Verma, 2015). However, no study has been carried out to determine the types and nutritive value of forage resources consumed by small ruminants in the Menoua division in the western region of Cameroon especially during the dry season. In addition, the use of these resources by pastoralists is irrational because of the variation in their nutritional values according to seasons and also less knowledge on various fodder dispersion in different agro-ecological zones (Awono, Njehoya and Akoa, 2012; Lemoufouet, Pamo and Tendonkeng, 2012; Tendonkeng *et al.,* 2013). Increased knowledge on the nutritional value of locally available fodder resources in a given agro-pastoral zone could enable the formulation of rations based on the nutritional needs of the animals.

Therefore, the general objective of the present study was to improve the knowledge on the small ruminants feeding strategies by developing a data bank on local fodder resources available in the dry season, together with their chemical compositions and nutritional values.

2. Materials and methods

Study area

The study took place in Menoua division of Cameroon with latitude/longitude coordinates: 5° 27' 0'' N/10° 4' 0'' E. The study was conducted from January to June 2021 for data collection in the field and for chemical composition analyses at the Animal Production and Nutrition Research Unit.

Material

The plant material consisted of fodder resources consumed by goats and sheep harvested from the herders' plots. The working tools consisted of data collection sheets, newspaper sheets for making the herbarium, a knife, a camera, tape and plastic bags.

Methods

The study was carried out in five (5) subdivisions of Menoua division, namely Dschang, Nkong-Ni, Penka-Michel, Fokoué and Santchou. Three (3) villages per subdivision were chosen in which small ruminant breeders were well represented. Fifty (50) breeders were interviewed to collect information on the types of fodder consumed by small ruminants, the parts generally taken and their preferences. With the permission of the farmers, animals were observed on the grazing area, the grazed resources and their phenological stage were recorded. The forages consumed were photographed, and 100 g sample of each fodder were collected for chemical composition analysis in the laboratory. Plants species were identified using Geerling's Woody Guide (Geerling, 1987), Van der Zon's Grasses of Cameroon (Van der Zon, 1992), Merlier and Montegut's Tropical Weeds (1982). Newsprint was used for species that could not be identified on the site and forwarded to the Cameroon National Herbarium for taxonomic identification.

Studied parameters

The parameters studied were as follows:

- Taxonomic identification, local name, preference and part of the plant consumed by small ruminants.
- The frequency of citation (FC) which reflects the regularity in the distribution of a species within a locality. It is expressed as the percentage of citations of a species in relation to the total number of people surveyed. The citation frequency of the fodder resources surveyed was calculated as recommended by Gbekley *et al.* (2015):

$$FC = n/N \quad \text{where,}$$

n: number of farmers citing the species,

N: total number of respondents.

- Chemical composition was determined according to the AOAC method (AOAC, 2016).
- The nutritive value of the herbaceous forage species listed (energy and nitrogen content) was estimated from the chemical composition (Van Soest, Robertson and Lewis, 1991), using the Equations of Vérité and Peyraud (1988), Jarrige *et al.* (1994) and Sauvant, Grenet and Doreau (1995).

Statistical analysis

The data were analysed and processed in an Excel spreadsheet version 2016 to calculate the chemical composition and nutritional value of forage. The SPSS 20.0 software was used for descriptive analysis of the data i.e. minimum, maximum, mean, standard deviation. Data on the chemical composition and nutritional value of fodder were subjected to an analysis of variance at the 5 percent threshold with the sub-divisions as a variation factor.

3. Results and discussion

In Menoua division, a total of 21 species belonging to 10 families were identified. The largest number of species recorded belonged to the family *Poaceae* (8), followed by *Fabaceae* (5) as presented in Table 1. In the families' *Lauraceae, Convolvulacea, Myrtaceae, Meliaceae, Euphorbiaceae, Asparagaceae, Asteraceae* and *Musaceae*, only one species was recorded.

Table 2 summarizes the chemical composition and nutritional values of the different forages. The most consumed parts by small ruminants on these fodders were the leaves; this observation is similar to that of Lucha and Chuyong (2016). This could be explained by the fact that during the dry season, the majority of certain forages are in their flowering stage and the leaves seem less lignified compared to other parts, namely the stems and roots. Breeders claimed that small ruminants consumed much more *Pennisetum purpureum*. This could be explained by its high availability, abundance and its high-water content compared to other species. Most farmers stated that animal preferences during the dry season are different from those of the rainy season. This is because given the scarcity of food resources during the dry season, they only consume available resources. This could explain the consumption of some species such as *Entandrophragma cylindricum, Draceana fragrans* and *Vernonia amygdalina* which were reported by a minority of herders and according to them, these species are not consumed during the rainy season.

The fibre content of *Poaceae* family ranged from 25.87 percent to 36.25 percent DM, that of *Fabaceae* family ranged between 13.69 percent and 23.31 percent DM, the protein content of *Poaceae* ranged between 4.72 percent and 24.15 percent DM, whereas that of *Fabaceae* family ranged from 25.02 percent to 27.82 percent DM. From Table 2, it can be seen that *Poaceae* had the highest levels of crude fibre, while *Fabaceae* had the highest levels of protein content; this statement is similar to that obtained by Boukila *et al.* (2006), Pamo *et al.* (2007) and Klein *et al.* (2013). The majority of the forages were taken at the flowering stage for chemical analysis. Energy requirements of ruminants are expressed in forage units. The present study revealed that the highest rate in milk and meat forage unit was recorded with *Manihot esculenta.* With the protein requirements expressed as digestible protein in the intestine, the highest rate of digestible protein was recorded with *Gliricidia sepium.*

Table 1: Species, family and local name of fodders resources

Families	Species	Local name	Common name
Poaceae	*Bracharia ruziziensis*	/	/
Poaceae	*Cynodon dactylon*	/	/
Poaceae	*Panicum maximun*	/	/
Poaceae	*Digiatria ciliaris*	/	/
Poaceae	*Pennisetum purpereum*	*mesouson, chouchoun*	Sussongo
Poaceae	*Trypsacum laxum*	/	/
Poaceae	*Imperata cylindrica*	*Keneum, fixtree*	/
Fabaceae	*Desmodium uncinatum*	/	Wedding force
Fabaceae	*Arachis glabrata*	/	/
Fabaceae	*Calliandra calothyrsus*	/	/
Fabaceae	*Leucena leucocephola*	/	/
Fabaceae	*Gliricidiasepium*	/	/
Lauraceae	*Persea america*	*Fehpiah*	Avocado leaf
Fabaceae	*Centrosema pubescens*	*Mecoop*	Wild bean leaf
Convolvulacea	*Ipomea batatas*	*Fehmetong*	Potato leaf
Myrtaceae	*Psidium guajava*	*Fehgoya*	Guava leaf
Meliaceae	*Entandrophragma cylindricum*	/	Sapelli
Euphorbiaceae	*Manihot esculenta*	*Fehkessala,*	Cassava leaf
Asparagaceae	*Draceana fragrans*	*Keun*	Tree of peace
Asteraceae	*Vernonia amygdalina*	*Mekan*	Ndole leaf
Musaceae	*Musa paradisiaca*	*Fehquiguien*	Banana leaf

Source: Authors' own elaboration.

Table 2: Chemical composition and nutritional values of forages

Species	Studied parameters											
	DM (%)	Ash (%DM)	OM (%DM)	CH (%DM)	FA (%DM)	CP (%DM)	CF (%DM)	Water content (%)	UFV (UF/100 kg DM)	UFL (UF/100 kg DM)	PDIN (g/kg DM)	PDIE (g/kg DM)
Bracharia ruziziensis	90.04	8.76	81.28	75.16	1.40	4.72	36.25	40.44	46.39	58.47	2.96	33.62
Cynodon dactylon	89.80	11.80	78.00	51.15	2.70	24.15	25.87	63.83	71.97	81.03	15.17	41.32
Digiatria ciliaris	89.26	17.49	71.77	47.75	1.80	22.22	27.07	57.78	68.12	77.51	13.95	36.95
Panicum maximun	90.40	10.20	80.20	64.69	3.01	12.6	31.04	67.41	60.39	70.79	7.91	41.13
Pennisetum purpereum	85.91	8.73	77.18	60.00	0.20	16.97	34.75	69.93	48.95	61.01	10.66	17.10
Imperata cylindrica	84.67	7.94	76.73	56.16	1.20	19.36	33.41	60.70	53.27	64.97	12.16	18.38
Trypsacum laxum	90.85	8.65	82.2	66.05	5.30	10.84	32.78	66.56	59.15	70.34	6.81	37.78
Gliricidia sepium	89.68	11.11	78.57	39.15	2.70	36.72	13.69	66.58	99.56	104.46	23.06	70.07
Arachis glabrata	90.40	9.60	80.80	52.78	3.00	25.02	23.31	57.78	78.03	86.14	15.71	51.94
Calliandra calothyrsus	90.79	6.01	84.78	57.66	0.70	26.42	16.80	59.35	89.62	95.17	16.59	80.66
Leuceana leucocephala	91.3	9.08	82.22	54.28	0.9	26.15	21.20	60.91	79.91	87.25	16.40	59.07
Desmodium uncinatum	83.87	7.85	76.02	48.38	2.40	25.24	21.96	66.59	80.27	87.85	15.85	54.24
Centrosema pubescens	88.27	9.34	78.93	42.61	8.50	27.82	18.36	75.34	95.79	102.34	17.47	66.55
Entandrophragma cylindricum	89.11	7.97	81.14	66.47	1.20	13.47	29.10	50.99	62.4	72.12	8.46	48.30
Ipomea batatas	89.33	8.34	80.99	56.11	4.84	20.04	26.02	78.86	74.01	83.00	12.58	49.49
psidium guajava	90.78	5.725	85.05	67.46	1.10	16.49	19.49	46.88	83.58	89.71	10.36	87.81
Manihot esculenta	89.26	12.06	77.20	37.89	9.88	29.44	16.40	70.54	101.92	107.83	18.48	70.53
draceana fragrans	88.45	11.48	76.97	45.71	5.86	25.4	26.44	70.70	74.64	84.11	15.95	36.39
Musa paradisiaca	90.35	10.97	79.38	54.58	1.40	23.39	24.83	67.34	72.63	81.19	14.69	47.65
vernonia amygdalina	89.26	12.06	77.20	42.23	5.54	29.43	16.38	52.75	96.63	102.36	18.48	70.53
Persea america	91.24	7.36	83.87	67.06	1.50	15.31	22.99	50.15	76.32	83.76	9.61	73.38

Source: Authors' own elaboration.

Note: DM: Dry matter, OM: Organic matter, CP: Crude protein, FA: Fatty acid, CH: Carbohydrates, CF: Crude fibre, UFV: Meat Forage Unit, UFL: Milk Forage Unit, PDIN: Digestible Protein In the Intestine allowed by Nitrogen, PDIE: Digestible Protein In the Intestine allowed by Energy.

4. Conclusions

In Menoua division, twenty-one (21) fodder species are used to feed small ruminants during dry season. These species belong to the families *Poaceae* (8), *Fabaceae* (5) *Lauraceae* (1), *Convolvulacea* (1), *Myrtaceae, Meliaceae* (1), *Euphorbiaceae* (1), *Asparagaceae* (1), *Asteraceae* (1) and *Musaceae* (1). Irrespective to the plant species, the most consumed parts by small ruminants on these fodders were the leaves.

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Presentation of some local fodders identified in Menoua Division, Western Highland of Cameroon

Source: Authors' own elaboration.

Bracharia ruziensis

Cynodon dactylon

Digiatria ciliaris

Panicum maximun

Pennisetum purpureum

Imperata cylindrica

Trypsacum laxum

Gliricidia sepium

Arachis glabrata

Leucaena leucocephala

Calliandra calothyrsus

Desmodium uncinatum

Centrosema pubescens

Entandrophragma cylindricum

Ipomea batatas

Psidium guajava

Manihot esculenta

Draceana fragrans

Musa paradisiaca

Vernonia amygdalina

Persea america

Utilization of various feedstuffs by small-scale beef cattle farmers under mixed farming systems in Egypt: A survey

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Abstract

This study aimed to characterize the adoption of various feeding packages (silage, hay, non-traditional green forages, urea and molasses) by the small-scale beef cattle farmers under mixed crop-livestock farming production systems in Egypt. A cross-sectional survey of 200 cattle farmers was carried out in four governorates, two in the Nile Delta region (Sharkia and Menoufia) and two in the middle Egypt region (El-Fayoum and Minya), through semi-structured interviews with questionnaires. Corn silage adoption was recorded with the highest frequency (39.5 percent) followed by hay (20.5 percent) while urea and molasses were adopted to only a limited extent (4 percent and 2 percent, respectively). The majority of farmers reported using corn silage for only one time and hay for more than one time. Significantly less ($P < 0.05$) live weight gain was reported for indigenous cows (0.48 kg/cow per day), crossbred cows (0.52 kg/cow per day), and buffaloes (0.59 kg/cow per day) by non-adopters of feeding packages than for adopters of the packages. Adopters of feeding packages reported significantly higher weight gain and finished weight for their cattle compared to non-adopters. Education of farmers seemed to have a positive association with adoption of feeding packages. Therefore, policymakers should try to reduce illiteracy among farmers, which reflects on the adoption rate.

Keywords

feeding packages, adoption, mixed farming system

1. Introduction

In Egypt, smallholder farming systems are characterized by having fewer than 11 heads of livestock per farmer (ASL2050-FAO AGA, 2018). Livestock play an important role in the livelihoods of people, especially the poor, as livestock production is a source of nourishment and employment for rural villagers. Animals are also a financial asset that can be liquidated in times of need and farmers use manure to fertilize their fields (Stür, Khanh and Duncan, 2013). Beef cattle play a major role as a protein source to the Egyptian population. In developing countries, population growth, changing consumer preferences, economic progress and urbanization are increasing demand for animal products (Delgado *et al*, 2021). In Egypt, per capita consumption of red meat was about 12.7 kg/year in 2012, which was lower than worldwide average (42.7 kg/year) (El-Sheemy *et al.*, 2015). The most widespread farming system practiced in Egypt is the mixed crop-livestock system where farmers use crop residues in making hay and silage to bridge feed scarcity during the summer period. In Egypt, intensive and semi-intensive systems comprise approximately 7 percent and 60 percent of the total bovine population, respectively.

Egypt has a relatively large population of ruminants, estimated at 18.247 million (MALR, 2015), that is affected by limited agricultural land and water resources. Feed affects livestock productivity, profitability, environmental impact, and human food and nutrition security. Animal feed is usually the biggest cost in livestock production, accounting for about 70 percent of the total variable costs

and in some cases even reaching up to 90 percent under intensive systems. (Makkar, 2016). Increases in the cost of production eventually result in higher retail prices of meat and milk. Adequate and good quality animal feed is important to optimize livestock productivity, and this is a big challenge for poor farmers. Therefore, it is necessary to cover animal nutritional requirements by optimising the utilization of crop residues, using livestock technology and innovation, that increase animal production. Practical and simple feeds (henceforth referred to as "feed packages") like hay, silage, urea, molasses and non-traditional green forages could easily be adopted by farmers to improve cattle productive efficiency and may help in reducing the overall cost of rations. Using such feed packages resulted in a 45 percent increase in the area planted with Egyptian clover (*Trifolium alexandrinum*) and a 19.8 percent increase in the cash crop area, and feeding costs were reduced by 29 percent and 44 percent for growing and fattening animals, respectively (El-Wardani *et al.*, 2005).

Beef cattle farmers in Egypt typically do not follow a scientific basis in feeding their animals. Most farmers do not purchase animal feed; animals are fed chiefly whichever forages and crop residues are produced on the farm. In winter there is a surplus of green forage, while in summer supply is short. Egyptian clover (Berseem) is considered the key forage crop in winter and corn leaves (Darawa) are the main forage in summer. Hay and straw are also common, particularly in summer, as well as variable quantities of concentrates. Optimizing the digestive use of poor-quality roughages, either by urea treatment or making silage, is a way to reduce the feeding costs. Therefore, whole maize silage and/or rice straw treated with ammonia or urea are used to improve quality and sustainable availability of forages across the year, resulting in more efficient production.

Smallholder beef cattle farmers in Egypt face many challenges, the most important one being the fluctuation in the availability of feed, which reflects on the low performance of the animals. The demand for animal-source protein is growing rapidly in Egypt due to rising population numbers and the current production fails to satisfy the demand for beef and achieve national self-sufficiently. Hence, meeting the growing Egyptian population's need for protein is a pressing challenge. At this point, to cover the current demand and increase the country's meat output, a judicious strategy focusing on improved feed packages may improve beef cattle production systems. This study was therefore undertaken to obtain baseline information on the current adoption feeding packages by beef cattle farmers.

2. Methodology

A cross-sectional survey of 200 households that raise cattle and were willing to participate in the survey was carried out through semi-structured interviews and questionnaires. The method of study sample selection can be classified among intentional or purposive sampling techniques. Research ethics were considered; the householders were informed about the purpose of the questionnaire and their participation in the study was voluntary.

The study was carried out in four governorates of Egypt, two in the Nile Delta region (Sharkia and Menoufia) and two in the middle Egypt region (El-Fayoum and Minya). Two different agro-climatic regions were selected according to the average temperatures to insure adequate geographical coverage of Egypt. The Delta region (30°N – 31°N) and Middle Egypt region (28°N – 30°N) have annual ranges in air temperatures from 27.6 to 31.2 °C and 29.5 to 33.2 °C, respectively (Abdrabbo, Farag and El-Desokey, 2015). From each governorate, 50 householders were chosen randomly. The questionnaire was initially tested in the field and then changes were made in sections that could be misunderstood by survey participants before extensive application. The data were collected with assistance of the extension staff belonging to the directorate of agriculture in each governorate. The collected data included basic information about social characteristics of the farmers, educational level of household members, cattle fattening periods, animal body weights at the beginning and end of fattening, weight gain, and types of feeding packages and agricultural by-products available in the study area. The farmers were asked whether they had adopted some of the common feeding packages (i.e. silage, hay, untraditional green forages, urea and molasses) on their farms.

In the present study qualitative and quantitative approaches were used. Categorical data were analysed using Chi-Square (Snedecor and Cochran, 1989), whereas the General Linear Model (GLM) procedure of SAS (SAS, 2010) was used to analyse the variation of numerical data. The fixed model used in the analysis was:

$$Y_{ijkl} = \mu + R_i + G_j + P_k + e_{ijkl},$$

where Y_{ijkl} is the value of the respective dependent variable, μ is the overall mean of the respective dependent variable, R_i is the effect due to the ith region, i = 1, 2 (1 = Nile Delta, 2 = Middle Egypt), G_j is the effect of the jth governorates (j = 1, 2,…4), P_k is the effect of the kth packages, (k =1, 2,… 6), e_{ijkl} is a random error associated with the ijkth observation and is assumed to be independently and normally distributed.

3. Results and discussions

Consistent with Egypt in general, the crop-livestock system was the prevailing production system employed by the households that were surveyed. Nutritional technological change has been the major powerful force for increasing animal productivity and boosting animal development (Balehegn *et al.*,2020). Educated farmers were thus expected

to be more frequent adopters of new technologies. Middle Egypt had a greater percentage of illiterate householders (32 percent) than the Nile Delta region (22 percent) and the lowest share of tertiary education (university graduates) was found in the Minya governorate (22 percent) while the highest percentage (50 percent) was in the Menoufia governorate (Table 1). Illiteracy was linked to poverty rate, as poverty rate (data not shown) is higher in Middle Egypt than in the Nile Delta. There is a high correlation between poverty and illiteracy (UNDP, 2008). High illiteracy may be due to deficiency of understanding, awareness and support by society regarding the importance of inclusive education requirements and many need to be addressed through advocacy and dialogue at the regional level.

Table 1: Socioeconomic analysis of the householders under the studied governorates and regions

Item	Sharkia		Menoufia		El-Fayoum		Minya		Nile Delta		Middle Egypt		Total	
	N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Education level														
Tertiary education	16	32	25	50	22	44	11	22	41	41	33	33	74	37
Secondary education	20	40	10	20	11	22	11	22	30	30	22	22	52	26
Read and write	5	10	2	4	6	12	7	14	7	7	13	13	20	10
Illiterate	9	18	13	26	11	22	21	42	22	22	32	32	54	27
Occupation														
Farmer	19	38	19	38	33	66	26	52	38	38	59	59	97	48.5
Employee	22	44	27	54	10	20	21	42	49	49	31	31	80	40
Other jobs*	9	18	4	8	7	14	3	6	13	13	10	10	23	11.5

Source: Authors' own elaboration.

Notes: a Differences between governorates for education levels were significant ($X^2 = 104.1383$, $P < 0.0001$). No significant difference between governorates for occupation ($X^2 = 49.6218$, $P < 0.0649$). Differences between regions for education levels were significant ($X^2 = 9.22$, $P = 0.0264$). No significant difference between regions for occupation ($X^2 = 0.06$, $P = 0.8025$).

b * Artisans and traders.

As a whole, about 37 percent of households had a tertiary education, 26 percent had a secondary education, 10 percent of them had the ability to read and write, while 27 percent were illiterate. It may be expected that the level of education is related to decision making of an owner and would contribute to their ability for efficient management in their farms. Also, education may positively affect the farmers' access to information that may help them increase their productivity. Ndanitsa (2008) stated that level of education determines the skill of farmers and how well informed they are to the innovations and technologies around them. Oladipo and Adekunle (2010) added that individuals with higher educational attainment are usually faster adopters of innovation.

Nearly 44 percent and 54 percent of the householders in Sharkia and Menoufia, respectively, had permanent professions as employees off-farm and worked on their farms as a secondary job. On the other hand, the majority of the householders in Minya (52 percent) and El-Fayoum (66 percent) worked on their farms exclusively and earned their living from agriculture. The same trend was observed in Middle Egypt, as approximately two thirds of the population of Upper Egypt is engaged in agriculture (Mohamed, 2015). As a whole, approximately half of the householders (49 percent) worked in agriculture as farmers (i.e. have no job out of farm), and around 40 percent and 11 percent, respectively, were farming part time and also worked as an employee or performed other jobs, respectively.

Adoption of feeding packages

Farmers depended mostly on the forage and crop residues produced on their lands. Fodder storage is crucial in guaranteeing that animals have enough feed all over the year.

The vast majority of farmers had fully adopted some of the feeding packages (Table 2). Corn silage adoption recorded the highest percentage (39.5 percent) followed by hay (20.5 percent). Corn silage was greatest in Menoufia and Minya, 54 percent and 38 percent, respectively. The highest percentage of hay adopters (34 percent) was found in Sharkia governorate. On the other hand, El-Fayoum had the highest percentage of non-traditional green forage adopters (32 percent). Urea and molasses were adopted by the farmers to a limited extent (4 percent and 2 percent, respectively) (Table 2). The likely reasons urea treatment is not used on a wide scale are because of inadequate extension efforts to popularize the technology, and unavailability of cash to purchase urea. Similar findings were reported by Nguyen (2004).

Due to inadequate extension education, farmers may lack knowledge about the utilization and advantages of these feeding packages, and sometimes they are not even aware of these packages. Moreover, uses of molasses are very limited due to problems related to transport and

Table 2: Adoption status of feeding packages under the studied regions and governorates

Feed packages	Nile Delta				Middle Egypt				Total	
	Sharkia		Menoufia		El-Fayoum		Minya			
	N	(%)	*N*	(%)	*N*	(%)	*N*	(%)	*N*	(%)
Corn silage	17	34	27	54	16	32	19	38	79	39.5
Hay	17	34	11	22	5	10	8	16	41	20.5
NGF*	7	14	2	4	16	32	9	18	34	17
Urea	2	4	1	2	4	8	1	2	8	4
Molasses	2	4	1	2	1	2	1	2	5	2.5
Non adopters	5	10	8	16	8	16	12	24	33	16.5

Source: Authors' own elaboration.

Notes: a Differences between region and governorates for adoption are significant ($X^2 = 200.00$, $P < 0.05$).

b * Non-traditional green forages

storage. Education somehow seemed to have a positive effect on feed package adoption because of the expected link between education and knowledge and the ability to read technical materials. Minya had relatively more non-adopters (24 percent) than Sharkia (10 percent). This may have been due to the higher illiteracy in Minya (42 percent) than Sharkia (18 percent). Paul *et al.* (2017) and Abdullah, Ali and Zamzuri Noor (2021) reported that level of education significantly affected cattle farmers on adopting technologies.

Frequency of adoption of feeding packages

Table 3 provides data on the number of times the various feeding packages were adopted by individual households, by governorate. In general, most packages have been used by farmers more than once, suggesting that farmers derived benefits from the packages. In Nile Delta, adoption of nutritional packages for one time only was more common in Sharkia (76.92 percent) than Menoufia (23.08). For Middle Egypt, single adoption occurred more often in El-Fayoum (66.67 percent) than in Minya (33.33 percent). The limited of adoption may be due to lack of local resources and the small herd size of the farmers (Kebebe, 2019). Smallholder farmers lack resources, so incentives to adopt feed technologies may increase their uptake. Extension systems are also weak, which means that farmers may lack awareness of the feeding packages and/or knowledge about how to adopt them properly. Improved understanding of the factors that facilitate or hinder adoption is crucial for eliminating such barriers.

Silage was the nutritional package most frequently adopted by farmers only one time, particularly in Sharkia. Perhaps the relative bulk (i.e. lower dry matter proportion) and perishability relative to the other packages caused more difficulties in transport and storage for some farmers.

Beef production

Results on animals subjected to various feed packages are in Table 4. The feeding different feed packages were associated with significant ($P < 0.05$) differences in fattening periods and live weight gains. The longest fattening period (360 days) was detected for indigenous and crossbred cows reared under non-adopters. The heaviest average finished weight of indigenous cows (400 kg) was observed for farmers who adopted urea and NGF, whereas the lightest finished weight (337.5 kg) was for cattle of non-adopters. Differences among feed packages were not significant. A similar trend was observed for crossbred cows and buffaloes. Some of the differences among feeding packages were significant, with urea providing the most favourable result. Correspondingly, indigenous cows, crossbred cows, and buffaloes of non-adopters all had the lowest live weight gain, being 0.48, 0.52, and 0.59 kg/cow per day, respectively (Table 4). These results agree with those reported by Allam *et al.* (2009) who found that feeding wheat straw treated with urea, molasses and enzymes to growing male goats significantly increased live weight gain. Ma *et al.* (1990) reported that responses in live weight gain of cattle to ammonization of wheat straw were significant. Cattle fed with corn silage had greater body weight gain than those not given silage (Nazli *et al.*, 2018).

Table 5 shows data on weight gain according to genotype and governorate. The longest fattening period (285 day) for indigenous cows was found in Menoufia, while the shortest one (220 day) was in Sharkia. Minya and Sharkia had the longest fattening periods for crossbred cows and buffaloes, i.e. 287 and 240 days, respectively.

Minya governorate had the heaviest start live weights for indigenous cows (208.67 kg) and crossbred cows (214.28 kg), although differences were not significant for all possible comparisons ($P < 0.05$). For buffaloes, the heaviest starting (213.0 kg) and finishing weights (447.47 kg) were found in Minya and Menoufia, respectively, although these differences were not significant from some other governorates.

Regarding the liveweight gains, the highest liveweight gains (kg/cow/day) for indigenous cows (0.82), crossbred cows (0.98), and buffaloes (1.09) were found in El-Fayoum

for the first case and Menoufia in the other two instances (Table 5). The difference from the governorate with the second highest daily gain was significant ($P < 0.05$) for crossbreds only.

Data showing fattening traits of beef animals in the studied regions are shown in Table 6. Middle Egypt recorded higher start weight, finished weight, and weight gain for indigenous cows (209.82 kg, 400.89 kg, and 0.84 kg/day, respectively). However, the values of starting liveweight (184.80 kg), finished liveweight (427.94 kg) and liveweight gain (1.02 kg/cow/day) for buffaloes were significantly higher in the Nile Delta region. The interpretation for this may be due to the dependence of farmers in the Nile Delta on buffaloes as a main source of income. This is reflected in the greater size of herds in Nile delta compared to those found in Middle Egypt (data not shown). Fahim *et al.*, (2018) had previously reported that buffalo herd sizes and productivity were higher in Nile Delta (EL-Behira, EL-Menoufia, EL-Qalubiya, EL-Sharkia and Kafr El Seihkh) than in Upper Egypt (EL-Fayoum, EL-Minya, Assiut, Beni-Suef and Sohag).

Table 3: Frequency of nutritional packages adoption by the farmers (N=167)

Items	Once		Twice		Thrice		Four times ≥	
	N	(%)	*N*	(%)	*N*	(%)	*N*	(%)
Nile Delta	**13**	**59.1**	**13**	**43.33**	**34**	**53.97**	**27**	**51.92**
Sharkia	**10**	**76.92**	**6**	**46.15**	**16**	**47.06**	**13**	**48.15**
Silage	8	80	2	33.33	4	25	3	23.08
Hay	2	20	3	50	6	37.5	6	46.15
NGF*	0	0	1	16.67	3	18.75	3	23.08
Urea	0	0	0	0	2	12.5	0	0
Molasses	0	0	0	0	1	6.25	1	7.69
Menoufia	**3**	**23.08**	**7**	**53.85**	**18**	**52.94**	**14**	**51.85**
Silage	3	100	5	71.43	12	66.67	7	50
Hay	0	0	2	28.57	4	22.22	5	35.71
NGF*	0	0	0	0	1	5.56	1	7.14
Urea	0	0	0	0	0	0	1	7.15
Molasses	0	0	0	0	1	5.55	0	0
Middle Egypt	**9**	**40.9**	**17**	**56.67**	**29**	**46.03**	**25**	**48.08**
Al-Fayoum	**6**	**66.67**	**11**	**64.71**	**14**	**48.28**	**11**	**44**
Silage	2	33.33	3	27.27	7	50	4	36.36
Hay	1	16.67	1	9.09	1	7.14	2	18.18
NGF*	2	33.33	6	54.55	4	28.57	4	36.36
Urea	1	16.67	1	9.09	1	7.14	1	9.1
Molasses	0	0	0	0	1	7.15	0	0
Minya	**3**	**33.33**	**6**	**35.29**	**15**	**51.72**	**14**	**56**
Silage	3	100	4	66.67	6	40	6	42.86
Hay	0	0	0	0	3	20	5	35.71
NGF*	0	0	1	16.67	5	33.33	3	21.43
Urea	0	0	0	0	1	6.67	0	0
Molasses	0	0	1	16.66	0	0	0	0
Total	**22**	**13.17**	**30**	**17.96**	**63**	**37.73**	**52**	**31.14**

Source: Authors' own elaboration.

Notes: a Differences between region governorates for repeated use of the package are significant ($X^2 = 262.2976$, $P < 0.05$).
b * Non-traditional green forages.

Table 4: Least square mean (M) ± standard error (SE) of fattening traits of beef animals under adopters and non-adopters of feeding packages

Item	Fattening period (days)	Start live weight (kg)	Liveweight at the end (kg)	Weight gain (kg/cow/day)
	M ± SE	M ± SE	M ± SE	M ± SE
Indigenous cows				
Silage	210.00±12.69^{b}	208.50±9.38^{ab}	396.25±9.64^{a}	0.89±0.02^{a}
Hay	232.50±21.02^{b}	200.00±18.89^{ab}	393.75±27.44^{a}	0.83±0.00^{ab}
NGF*	190.00±10.00^{b}	242.50±13.76^{a}	400.00±12.90^{a}	0.83±0.03^{ab}
Urea	320.00±40.00^{a}	166.66±16.66^{b}	400.00±28.86^{a}	0.74±0.04^{b}
Molasses	-	-	-	-
Non adopters	360.00±35.00^{a}	162.500±12.50^{b}	337.50±12.50^{b}	0.48±0.04^{c}
Crossbred cows				
Silage	236.12±13.03^{b}	205.00±8.87^{ab}	428.12±11.43^{a}	1.02±0.04^{a}
Hay	282.85±22.17^{ab}	197.14±22.32^{ab}	435.71±22.50^{a}	0.87±0.04^{bc}
NGF*	300.00±29.27^{ab}	197.14±25.51^{ab}	428.57±18.44^{a}	0.78±0.03^{d}
Urea	280.00±40.00^{ab}	233.33±44.09^{a}	483.33±44.09^{a}	0.92±0.11^{b}
Molasses	360.00±25.22^{a}	200.00±19.12^{a}	500.00±50.22^{a}	0.83±0.02^{c}
Non adopters	360.00±13.11^{a}	143.33±6.66^{b}	333.33±16.66^{b}	0.52±0.05^{c}
Buffalo				
Silage	204.68±21.43^{c}	167.68±17.09^{c}	389.28±34.16^{b}	0.98±0.05^{a}
Hay	230.16±36.70^{b}	176.83±30.04^{b}	392.00±54.07^{b}	0.92±0.03^{ab}
NGF*	200.66±50.20^{d}	151.00±85.73^{d}	317.66±57.99^{d}	0.83±0.04^{b}
Urea	180.00±0.00^{dc}	350.00±0.00^{a}	500.00±0.00^{a}	0.83±0.00^{b}
Molasses	360.00±50.13^{a}	150.00±40.39^{d}	450.00±23.12^{b}	0.83±0.04^{b}
Non adopters	140.66±71.46^{c}	167.66±82.33^{c}	251.33±24.50^{c}	0.59±0.03^{c}

Source: Authors' own elaboration.

Notes: a $^{a\text{-}b\text{-}c\text{-}d}$ Means, within the same column, with different superscripts differ significantly ($P < 0.05$).

b * Non-traditional green forages.

Table 5: Least square mean (M) ± standard error (SE) of fattening traits of beef animals in the studied governorates

Item	Fattening period (days)	Liveweight at beginning (kg)	Liveweight at the end (kg)	Liveweight gain (kg/cow/day)
	M ± SE	M ± SE	M ± SE	M ± SE
Indigenous cows				
Sharkia	220.00±20.00^{b}	188.88±13.88^{c}	355.55±13.02^{b}	0.78±0.04^{ab}
Menoufia	285.00±45.00^{a}	200.00±0.00^{b}	400.00±35.35^{ab}	0.72±0.10^{b}
Al-Fayoum	222.85±20.31^{b}	203.21±15.34^{ab}	382.14±11.25^{ab}	0.82±0.03^{a}
Minya	236.00±22.51^{ab}	208.67±20.23^{a}	393.33±17.16^{a}	0.79±0.05^{ab}
Crossbred				
Sharkia	268.23±21.89^{b}	207.64±19.06^{b}	444.11±18.63^{ab}	0.91±0.03^{b}
Menoufia	245.00±21.47^{c}	197.69±11.16^{bc}	400.00±25.31^{c}	0.98±0.03^{a}
Al-Fayoum	270.00±18.31^{b}	193.33±15.29^{c}	427.77±12.92^{b}	0.90±0.04^{b}
Minya	287.14±21.02^{a}	214.28±13.85^{a}	450.00±13.86^{a}	0.85±0.04^{c}
Buffalo				
Sharkia	240.11±28.11^{a}	182.47±21.77^{ab}	406.11±39.11^{a}	0.93±0.03^{b}
Menoufia	240.05±20.99^{a}	186.89±17.17^{ab}	447.47±26.24^{a}	1.09±0.02^{ab}
Al-Fayoum	107.77±45.25^{b}	109.44±46.26^{b}	223.33±87.49^{b}	0.96±0.05^{a}
Minya	217.75±40.59^{a}	213.00±34.60^{a}	412.62±61.66^{a}	0.86±0.03^{ab}

Source: Authors' own elaboration.

Note: $^{a\text{-}b\text{-}c}$ Means within the same column sub-section with different superscripts differ significantly ($P < 0.05$).

Table 6: Least square mean (M) ± standard error (SE) of fattening traits of beef animals in the studied regions

Item	Fattening period (days)	Liveweight at beginning (kg)	End liveweight (kg)	Liveweight gain (kg/cow/day)
	M ± SE	M ± SE	M ± SE	M ± SE
Indigenous cows				
Nile Delta	240.00±20.38^{a}	192.30±9.55^{b}	369.23±14.47^{b}	0.76±0.04^{b}
Middle Egypt	231.42±13.70^{b}	209.82±9.35^{a}	400.89±8.95^{a}	0.84±0.02^{a}
Crossbred cows				
Nile Delta	258.62±15.50^{b}	203.33±11.69^{Ns}	425.00±15.49^{b}	0.93±0.06^{a}
Middle Egypt	277.50±13.67^{a}	202.50±10.53^{Ns}	437.50±9.52^{a}	0.88±0.03^{b}
Buffalo				
Nile Delta	240.08±17.03^{a}	184.80±13.51^{a}	427.94±23.00^{a}	1.02±0.02^{a}
Middle Egypt	159.52±32.70^{b}	158.17±31.30^{b}	312.41±58.02^{b}	0.91±0.04^{b}

Source: Authors' own elaboration.

Note: $^{a\text{-}b}$ Means within the same column sub-section with different superscripts differ significantly ($P < 0.05$).

4. Conclusion and recommendations

Corn silage was the most-adopted feed package, followed by hay. However, a greater proportion of farmers adopted corn silage for only one time and hay for more than one time. Urea and molasses were adopted by the farmers only to a limited extent. Adopters of feeding packages recorded significantly higher weight gain and finished weights for their cattle than the non-adopters. This suggests that the feeding packages contain nutritional value that improves growth and condition of animals during the dry season, although other factors may have played a role. Literate farmers more frequently adopted feed packages, and this was accompanied by greater productivity of the animals fed, although causation could not be shown.

Policymakers should try to reduce the farmers' illiteracy rate, which would presumably have a positive effect on the adoption rate of feed technologies. As a complementary approach, agriculture extension specialists should simplify the information so that it is more easily understood by the literate and undereducated farmers. Raising awareness about feed packages, providing technical support and tailored training are potential ways to spread information about a suitable technology and increase adoption rate. Such activities should be based on a bottom-up approach taking into consideration of the needs, objectives and abilities of the poor smallholder farmers.

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Growth performance and digestibility of new mutant lines of sorghum in Indonesia

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Abstract

The suitability of sorghum as animal forage has been widely studied in Indonesia. In the last few years, National Nuclear Energy Agency of Indonesia (BATAN) has developed G5 and G8 as new mutant lines of sorghum for ruminant forage. This study aimed to determine the growth performance and digestibility of G5 and G8 lines. Two existing varieties and the two mutant lines were examined in our study. Numbu variety was used as a national control, Pahat variety was used as a mutant variety control, and G5 and G8 were the new mutant lines. Growth performance, nutrient composition and *in vitro* digestibility were observed in this study. The G5 sorghum mutant lines had highest dry matter yield ($p < 0.01$). Numbu was the tallest cultivar ($p < 0.01$). Variety G5, as a brown midrib (BMR) type, had the lowest neutral detergent fibre and acid detergent fibre values ($p < 0.01$). Furthermore, G5 also had the highest non-fibre carbohydrate value ($p < 0.01$). As a green midrib type, G8 contained the highest crude protein content ($p < 0.01$). The *in vitro* digestibility of total mixed rations based on Pahat and G5 sorghum forages showed the highest dry matter and organic matter digestibility ($p < 0.01$). Breeding programmes using the radiation mutation technique in Indonesia can improve growth performance and digestibility of sorghum. As a BMR type, G5 mutant lines provide high biomass yield and digestibility. G8 mutant lines contain high crude protein value. Therefore, we can suggest both of them to be used as forage sources in dry areas of Indonesia.

Keywords

digestibility, growth performance, mutant lines, sorghum

1. Introduction

Several livestock producing areas in Indonesia have a dry climate, including in Sulawesi, East Nusa Tenggara and Yogyakarta. Appropriate forage management is necessary to anticipate this challenge. Sorghum (*Sorghum bicolor*) is a suitable forage to cultivate in extreme environments. Forage sorghum is an important forage source in arid and semiarid regions (Perazzo *et al.*, 2017). Sorghum is an annual or perennial plant that uses water more efficiently than corn (Al Khalasi *et al.*, 2010; Astigarraga *et al.*, 2014). The suitability of sorghum as an animal forage has been widely studied in Indonesia. Moreover, the development of forage sorghum in Indonesia is mainly based on the (radiation-induced) mutation breeding technique by the National Nuclear Energy Agency of Indonesia (BATAN) (Sriagtula *et al.*, 2017; Sajimin *et al.*, 2018; Wahyono *et al.*, 2019b).

In the last few years, BATAN has developed G5 and G8 as new mutant lines of sorghum for ruminant forage (Wahyono *et al.*, 2019b). Based on leaf midrib and stem types, G5 is a brown midrib (BMR) type variety, while G8 is a green midrib (GMR) type (Wahyono *et al.*, 2019a). Growth performance and nutritive value for ruminant forages are affected by different types of sorghum. Therefore, this study aimed to determine the growth performance and digestibility of G5 and G8 as promising new mutant lines. This experiment was necessary to provide information about the performance of sorghum-based rations for ruminants that can be applied in the dry areas of Indonesia.

2. Materials and methods

This study was conducted in the field laboratory and the animal nutrition laboratory of the Center for Isotope and Radiation Application (CIRA), National Nuclear Energy Agency of Indonesia (BATAN), National Research and Innovation Agency Republic of Indonesia (BRIN), Jakarta, Indonesia.

Planting and growth performance measurements

Two control varieties and two new sorghum mutant lines were examined in our study. Numbu variety was the national control; Pahat variety was the mutant variety control; G5 and G8 were the new mutant lines. Numbu and Pahat are white midrib (WMR) type varieties. Each cultivar was planted in a 20 × 60 cm planting area at a 5 cm depth. Two applications of fertilizers were used. The first application consisted of urea, trisodium phosphate and potassium chloride at the ratio of 2:3:2 (g/g/g) at 210 kg/ha. The second application was simply urea, at 140 kg/ha. Plants were harvested at the hard dough stage (115 days after sowing). Dry matter (DM) yield, plant height, stem diameter, leaf length, leaf width and stem:leaf:panicle ratio were recorded as growth performance parameters. Organic matter, crude protein (CP), ether extract, non-fibre carbohydrate, neutral detergent fibre and acid detergent fibre contents were obtained from the previously published experiment of Wahyono *et al.* (2019).

In vitro Digestibility Measurements

The *in vitro* digestibility test was conducted by using the Rumen Simulation Technique (RUSITEC) (Czerkawski, 1986; Kajikawa *et al.*, 2003) for total mixed rations (TMR) based on sorghum forage (Pahat, G5 and G8 only) and concentrate (60 percent:40 percent DM composition; 97 g/day crude protein and 481 g/day total digestible nutrients). Numbu variety was not observed because it has lower nutrient content than the other three cultivars. Rations were incubated for 14 d. Dry matter digestibility (DMD), organic matter digestibility (OMD), total gas production, CO_2:CH_4 total gas ratio (Wahyono *et al.*, 2019b), total volatile fatty acids (TVFA) (Kromann, Meyer and Stielau, 1967) and N-ammonia (Conway, 1951) products were analysed in this study. The DM and OM residual substrate samples were collected every 48 h incubation. Total gas was collected into gas bag (5 l SANSHIN®) every 24 h incubation. Total gas volumes were measured with a gas meter. Methane and CO_2 concentration were measured by MRU VarioPlus *gas analyzer*. A liquid effluent was collected into a glass bottle each 24 h and taken for TVFA and N-ammonia measurements.

Data analysis

The data was analysed using a randomized block design with five replicates. Differences among treatments were analysed using Duncan's Multiple Range Test (DMRT) (Steel, Torrie and Dickey, 1997) which was calculated by using SPSS version 22.0. The variation among treatments for growth performance, nutrient composition and *in vitro* digestibility were tested at 0.01 significance level or 99 percent confidence level.

3. Results and discussion

Except for leaf ratio, significant differences ($p < 0.01$) were observed for all growth performance parameters among these four sorghum varieties (Table 1). The G5 sorghum mutant lines had the highest DM yield ($p < 0.01$). Numbu, the national control variety, was the tallest sorghum cultivar ($p < 0.01$). It is interesting to note that Pahat, as a grain type sorghum (Wahyono *et al.*, 2019b), had the highest panicle ratio and lowest stem ratio. High biomass is a major consideration in breeding programmes based on mutations via radiation. A previous study reported that BMR sorghum types tended to produce higher biomass yield than conventional sorghum, but the difference with staygreen/GMR types was not significant (Vietor, Rhodes and Rooney, 2010). On the contrary, Li *et al.* (2015) demonstrated that there were no significant differences in DM yield among BMR, GMR and white midrib (WMR) sorghum varieties. The differences in results among studies may be due to the differences of climate and organic material of the soil (Sajimin *et al.*, 2018).

As reported in Table 2, G5, as a BMR type, had lower neutral detergent fibre (NDF) and acid detergent fibre (ADF) values than Numbu ($p < 0.01$). Furthermore, G5 also had highest non-fibre carbohydrate (NFC) value ($p < 0.01$). Based on its general characteristics, BMR sorghums usually have lower lignin content than conventional sorghum (Li *et al.*, 2015; Sriagtula *et al.*, 2017; Wahyono *et al.*, 2019b). This usually equates to high digestibility, as shown in Table 3 for G5 (BMR). Lignin is part of the ADF content. Acid detergent fibre has a negative correlation with forage digestibility (Li *et al.*, 2015; Wahyono *et al.*, 2019b). As usual for a GMR type, G8 contained the highest CP content (Table 2) ($p < 0.01$). These characteristics would support the expectation of greater availability of protein derived from forage based on G8.

Table 1: Growth performance of the tested sorghum cultivars

Parameters	Cultivars*			
	Numbu	Pahat	G5	G8
Dry matter yield (ton ha^{-1})	25.21±0.57^{a}	26.09±0.26^{b}	26.88±0.61^{c}	25.28±1,03^{a}
Plant height (cm)	305.44±3.91^{c}	124.00±3.91^{a}	237.22±9.63^{b}	235.89±3.33^{b}
Stem diameter (mm)	16.73±0.86^{b}	22.97±1.50^{d}	17.73±1.36^{b}	14.83±0.56^{a}
Leaf length (cm)	105.89±2.20^{c}	93.67±3.74^{b}	92.22±3.63^{b}	83.78±3.60^{a}
Leaf width (cm)	8.00±0.23^{b}	8.48±0.48^{b}	8.38±0.52^{b}	7.08±0.32^{a}
Ratio (%)				
Stem	63.10±2.00^{b}	48.48±3.03^{a}	60.60±1.94^{b}	61.06±2.01^{b}
Leaf	15.94±1.29^{a}	17.23±2.37^{a}	16.54±2.32^{a}	17.66±1.02^{a}
Panicle	20.97±2.67^{a}	34.30±2.87^{b}	22.86±1.71^{a}	21.28±0.99^{a}

Source: Authors' own elaboration.

Notes: a * Numbu = national variety in Indonesia; Pahat = existing variety in Indonesia from mutation breeding technique; G5 and G8 = new mutant cultivars.
b ^{a,b,c} Means with different superscripts within rows are different ($p < 0.01$).

Table 2: Nutrient fractions of the tested sorghum cultivars

Parameters	Cultivars*			
	Numbu*	Pahat**	G5	G8
Organic matter	94.70±0.53^{b}	89.66±0.62^{a}	94.42±0.44^{b}	93.80±0.95^{b}
Crude protein	7.89±0.17^{a}	8.32±0.30^{b}	8.48±0.26^{b}	9.19±0.49^{c}
Ether extract	2.46±0.38^{b}	2.11±0.10^{a}	2.03±0.12^{a}	2.10±0.15^{a}
Non fibre carbohydrate	32.56±0.93^{a}	34.54±1.91^{bc}	38.79±1.92^{d}	36.65±2.18^{cd}
Neutral detergent fibre	52.78±0.67^{b}	44.70±1.53^{a}	45.11±1.84^{a}	45.86±1.37^{a}
Acid detergent fibre	31.93±0.51^{d}	24.14±0.91^{a}	26.14±0.46^{b}	27.97±0.93^{c}

Source: Wahyono, T., Sugoro, I., Jayanegara, A., Wiryawan, K.G. & Astuti, D.A. 2019b. Nutrient profile and in vitro degradability of new promising mutant lines sorghum as forage in Indonesia. *Advances in Animal and Veterinary Sciences*, 7(9). https://doi.org/10.17582/journal.aavs/2019/7.9.810.818

Notes: a * Numbu = national variety in Indonesia; Pahat = existing variety in Indonesia from mutation breeding technique; G5 and G8 = new mutant cultivars.
b ^{a,b,c,d} Means in the same row with different superscripts within rows are different ($p < 0.01$).

Table 3: Average of digestibility of total mixed rations (TMR) based on the sorghum cultivars by *in vitro* continuous rumen culture (n = 42; t = 14 d)

Parameters	Cultivars in TMR*			SEM
	Pahat	G5	G8	
Dry matter digestibility (%)	45.20^{b}	45.21^{b}	42.42^{a}	0.366
Organic matter digestibility (%)	47.99^{b}	46.85^{b}	44.95^{a}	0.355
Total gas production (ml/15 g DM)	880.71^{a}	932.86^{a}	800.71^{a}	37.384
CO_2:CH_4 gas ratio	4.31^{a}	6.73^{b}	7.20^{b}	0.330
Total volatile fatty acids (mM)	116.71^{a}	135.43^{b}	121.07^{a}	2.826
N-ammonia (mg/100 ml)	7.74^{a}	9.01^{a}	8.22^{a}	0.289

Source: Authors' own elaboration.

Notes: a * Numbu = national variety in Indonesia; G5 and G8 = new mutant cultivars.
b ^{a,b} Means with different superscripts within rows are different ($p < 0.01$).

The digestibility and rumen fermentation characteristics of TMR based on sorghum forage are presented in Table 3. Pahat and G5 showed highest digestibility of DM and organic matter ($p < 0.01$). The lower NDF and ADF fractions in G5 explain the higher digestibility values relative to G8. The Pahat variety has a high panicle ratio, which increases the non-structural carbohydrate fraction and improves digestibility (Wahyono *et al.*, 2019b). Kondo *et al.* (2015) stated that high ratio of non-structural carbohydrates, including soluble carbohydrates, could increase feed digestibility. High CO_2:CH_4 ratio relates to high efficiency of nutrient utilization. Total mixed rations

based on Pahat forage had the lowest CO_2:CH_4 ratio ($p < 0.01$). Similarly, in a single forage screening study (Wahyono *et al.*, 2019b), G5 and G8 mutant lines had lower CH_4 emissions than Pahat (Wahyono *et al.*, 2019b). High CH_4 production indicated a lot of energy losses in form of gas (Sugoro *et al.*, 2015). The highest TVFA concentration was produced by TMR based on G5 sorghum ($p < 0.01$). The various results suggest that the fibre source from G5, as a BMR type, was easily degradable to support rumen microbial population.

4. Conclusions

As a BMR type, the G5 mutant line produced high biomass yield and digestibility, whereas G8 mutant lines contained higher CP value than other cultivars tested. We can suggest both of these varieties to be used as a forage source in dry areas of Indonesia when fed as part of a properly balanced diet.

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Multi trait genetic evaluations of dairy cattle crossbred progeny in Sri Lanka for milk and constituents using test day model approach

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Abstract

A study was carried out for multiple trait estimation of genetic parameters and breeding values of dairy cattle on six traits (milk fat and protein yields, fat percent, protein percent, and electrical conductivity (EC) in milk) based on test day (TD) data in 3 districts of Sri Lanka. The TD records were obtained by manual recording and by using a Lactoscan® analyser for visits to all large government farms (> 150 cows) and 87 smallholder farmers of 3 districts on monthly basis. After editing, a set of 2 109 test day records with complete information on pedigree, birth date, parity, calving date, lactation length and parity of the animals with at least 3 TD data were used for Restricted Maximum Likelihood analyses. A repeatability TD model (RPM) and a random regression model (RRM) based on Legendre polynomials (LP) were compared using Akaike's information criterion (AIC) and log likelihood ratio test (LRT). Herd-calving year-month group (fixed), TD group (random), and fixed LP regressions on age at calving and days in milk (both nested within parity) were used in both models. In addition, the RPM included random animal and permanent environment (PE) effects while the RRM included third-order LPs for random animal and PE effects. Heritability estimates for the six traits under the RPM were 0.28, 0.21, 0.28, 0.13, 0.38 and 0.002, respectively. The EC showed negative genetic correlations with yield traits but positive estimates with constituent percentages. Sire rank correlations were significantly positive between the two models for all traits except for protein yield ($p < 0.05$). With lower AIC and significant LRT ($p < 0.05$), the RRM could be recommended for future genetic evaluations of test day data at the field level in Sri Lanka. Test day recording is essential to obtain data on milk component yield at field level where smallholder farmers have no recording equipment. The genetic correlation structure among traits and sire rankings shows the need for implementing an index-based selection to improve the economic traits simultaneously. With a superior fit, random regression model can be recommended for test day animal model evaluations of dairy cattle for the above field farm data scenarios.

Keywords

correlations, dairy cattle, genetic evaluations, heritability, model comparisons, random regression

1. Introduction

Performance recording and genetic evaluation programmes at the national level for economic traits are essential for selection of candidate dairy bulls and potential dams for various climatic zones and management scenarios

in Sri Lanka (Silva and Dematawewa, 2020). At present, daily milk recording takes place in relatively large scale (> 150 cows) state dairy farms, but rarely at the individual producer level, which comprises of about 90 percent of the national herds (Perera and Jayasuriya, 2008). Even in large herds, recording of milk constituent percentages takes place only when selling on a bulk tank basis where pricing is based on fat and solid-non-fat percentages. Thus, an island wide test-day (TD) recording scheme, with trained recorders (with milk analysers) visiting farms on monthly basis, is essential to obtain field level information on milk constituent yields. In 2010, Ministry of Livestock and Rural Development of Sri Lanka (MLRD) launched an FAO funded pedigree and performance recording scheme (PPRS) with a TD recording programme in several districts involving over 1 000 individual producers and several state farms (Silva and Dematawewa, 2020). Milk records (morning and evening milking) and their constituent percentages were collected using Lactoscan® (Nova Zagora, Bulgaria) analysers by visiting the farms monthly. In parallel, a TD recording and performance evaluation project was initiated in 2016 through an IAEA (Vienna) funded CRP project (D31028) on genome enabled selection that enabled multiple trait genetic evaluation of dairy cattle at field level using TD models (IAEA, 2015). The evolution and advantages of various TD model evaluations have been reviewed extensively by Bilal and Khan (2009), the main advantage being the ability to handle temporal non-genetic variations of individual TDs. The objective of this study was to conduct multi-trait genetic parameter estimation and best linear unbiased prediction (BLUP) evaluations of artificial insemination (AI) bulls and their offspring using a repeatability TD model (RPM) and compare its sire ranking and goodness of fit with random regression model (RRM) evaluations.

2. Materials and methods

This study involved the dairy farms participating in the government's PPRS TD recording programme in Anuradhapura, Polonnaruwa and Kurunegala districts (Silva and Dematawewa, 2020). All farms practiced semi-intensive management with some concentrate supplementation in cattle sheds. Among the information recorded, herd, animal identification number (ID), sire and dam IDs, birth date, calving date, age at calving, parity, and TD records were used for this analysis. Two calving seasons (*Yala*: Sept-Feb and *Maha*: Mar-Aug) were defined based on two monsoon seasons and moving averages. Dams of the cows were purebreds or crossbreds of Friesian, Jersey, Sahiwal and Australian Friesian Sahiwal breeds while all AI sires in the final dataset were Jerseys. Fat percent, protein percent and electrical conductivity (EC) of milk were measured on TD using a Lactoscan® milk analyser. The EC (mS/cm) was treated as an indicator of mastitis (Goodling *et al.,* 2000; Jamrozik *et al.,* 1997). Only the cows with a minimum of 3 TD records were considered for the analysis. Test-days were categorized into 4–month groups. Five parity groups were formed as 1, 2, 3, 4, and > 4. Age at calving varied from 23.87 to 153.7 months. Adjacent herds were clustered when the herd size was insufficient and contemporary groups were formed using herd mates that calved in the same year and season (HYS). Lactation length was truncated at 305 days (Jakobsen *et al.,* 2002). After editing, a set of 2109 TD records of 191 cows of 13 sires with complete information were used for the analysis. Days in milk (DIM_t where t = 4, …, 305) was converted to q_t by using $q_t = (DIM_t - DIM_{min})/(DIM_{max} - DIM_{min})$ so that $-1 < q_t < +1$ and were used in Legendre polynomials (LP) as in (Jakobsen *et al.,* 2002). The mth order LP ($\emptyset_m$) was defined as:

$$\emptyset_m(t) = \frac{1}{2^m}\sqrt{\frac{2m+1}{2}} \cdot \sum_{n=0}^{[m/2]} (-1)^n \binom{m}{n}\binom{2m-2n}{m} (t)^{m-2n}$$

A similar conversion was performed for calving age to obtain p_w where $-1 < p_w < +1$. Finally, the following six trait RPM was used for variance component and breeding value estimation (Adnøy, Nævdal and Svendsen, 2000):

$$Y_{ijklwtn} = HYS_{in} + TD_{jn} + Parity_{kn} + \sum^{2}_{m=0} a_{kmn}\emptyset_m(p_w) + \sum^{3}_{m=0}\beta_{kmn}\emptyset_m(q_t) + animal_{ln} + pe_{ln} + e_{ijklwtn}$$

where, $Y_{ijklwtn}$ is the jth TD record on nth trait for the lth cow in ith HYS group (fixed) and kth parity group (fixed). The effect of jth test day on nth trait (TD_{jn}) was treated as random (Andonov *et al.,* 2013). The a_{kmn} and β_{kmn} were fixed regression parameter estimates of the nth trait for calving age and days in milk nested within parity, respectively with respect to the mth LP. Additive genetic and permanent environmental (PE) effects of lth cow for nth trait are $animal_{ln}$ and pe_{ln}, respectively; and $e_{ijklwtn}$ is the random residual.

Restricted Maximum Likelihood estimates for (co) variance components (TD, animal, PE and error) were obtained using REMLF90 software and subsequently breeding values for sires, dams and cows were obtained using BLUPF90 software (Misztal *et al.,* 2018). Heritability was estimated as $h^2 = \sigma^2_{animal}/(\sigma^2_{animal} + \sigma^2_{pe} + \sigma^2_e)$. Sire ranks were compared with respect to the six traits.

Alternatively, the following random regression model (RRM) was fitted using REMLF90 program for comparison in terms of the best fit and sire rankings (Andonov *et al.*, 2013):

$$Y_{ijklwt} = HYS_i + TD_j + Parity_k + \sum^{2}_{m=0} a_m\emptyset_m(p_w) + \sum^{3}_{m=0}\beta_m\emptyset_m(q_t) + \sum^{3}_{m=0} a_{lm}\emptyset_m(q_t) + \sum^{3}_{m=0} pe_{lm}\emptyset_m(q_t) + e_{ijklwt}$$

where, a_{lm} and pe_{lm} are the random regression coefficients for additive genetic and PE effects of lth cow for the mth LP, respectively. Log likelihood ratio (LRT) and Akaike's information criterion (AIC) were used for model

comparison and Spearman's rank correlation was used for comparison of sire rankings.

3. Results and discussion

After final edits of the initial data set, only 2109 TD records of 402 cows with complete pedigree data could be used for multiple trait analysis. Although all cows were crossbreds of Jersey sires, most were maintained at the smallholder level under suboptimal climatic and management conditions. Hence, the mean milk, fat and protein yields (Table 1) were much lower than European results (Jamrozik *et al.*, 1997) but comparable with the findings of studies under similar tropical conditions (Bilal, Khan and Bajwa, 2008).

The RPM model estimates (Table 2) showed that substantial amount of genetic variation exists in the dairy population. Similar to (Padilha *et al.*, 2015), high PE estimates in Table 2 indicate the large non-genetic variation among TDs within a lactation, which is inherent to small herds. The resulting heritability estimates are similar to those of Danish Holsteins (Jakobsen *et al.*, 2002), but much higher than those reported for Sahiwals in Pakistan (Bilal, Khan and Bajwa, 2008). The EC showed a very low heritability estimate compared with findings in (Goodling *et al.*, 2000), indicating the trait, which is related to somatic cell score and mastitis, is very much influenced by the management measures than genetics.

The positive genetic correlations among milk fat and protein yields in Table 3 and negative relationships between milk yield and constituent percentages are similar to the results of (Jamrozik *et al.*, 1997).

Goodling *et al.* (2000) reported a numerically positive relationship between EC and somatic cell score and Jamrozik *et al.* (1997) found negative relationships between somatic cell score and yield traits. Similarly, Table 3 indicates that high yielding cows tend to have a genetic tendency for decreased EC. This suggests a smaller prevalence of subclinical mastitis, but this result may not be very meaningful, given the very small heritability of EC.

Table 1: Descriptive statistics of the traits based on test day records

Trait	Records (*n*)	Mean	Minimum	Maximum	SD
305-day Milk yield (kg)	2 109	6.61	1.00	20.70	2.78
Fat content (%)	2 109	3.41	0.83	7.15	0.60
305-day Fat yield (kg)	2 109	0.22	0.01	0.66	0.10
Protein content (%)	2 109	3.13	0.10	4.63	0.34
305-day Protein yield (kg)	2 109	0.21	0.01	0.77	0.09
Electrical conductivity (mS/cm)	2 109	6.05	0.10	6.50	0.09

Source: Authors' own elaboration.

Table 2: Genetic parameter estimates of 305-day milk, fat and protein yields, fat percent, protein percent and electrical conductivity based on the repeatability model

Trait	Parameter estimates			
	Additive	Permanent environment	Error	Heritability (%)
305-d Milk yield	2.229	2.808	4.500	28.337
Fat %	0.048	0.196	0.162	12.861
305-d Fat yield	0.002	0.004	0.006	20.614
Protein %	0.044	0.014	0.089	38.751
305-d Protein yield	0.002	0.003	0.005	28.459
Elect. conductivity	0.0003	0.0001	0.148	0.202

Source: Authors' own elaboration.

Table 3: Estimates of genetic (upper diagonal) and phenotypic correlations (lower diagonals) among 305-day milk, fat, and protein yields, fat percent, protein percent, and electrical conductivity (EC) under repeatability model

	Correlation coefficients					
	Milk yield	Fat %	Fat yield	Protein %	Protein yield	EC
Milk yield		-0.011	0.941	-0.735	0.998	-0.616
Fat %	-0.059		0.306	0.452	0.037	0.556
Fat yield	0.921	0.302		-0.551	0.954	-0.376
Protein %	-0.073	-0.011	-0.067		-0.688	0.887
Protein yield	0.964	-0.057	0.889	0.174		-0.573
EC	0.039	0.039	0.043	0.057	0.056	

Source: Authors' own elaboration.

For RRM, Schaeffer (2004) showed that heritability can be calculated for regression coefficients of LP and stated that it is perhaps the best way to look at heritability in an RRM, rather than estimating variances for every day in the test period. Table 4 shows that the regression coefficient of first order LP (a_1) had higher heritability estimates than the other coefficients. The percentage of PE component (Table 5) seems much greater than additive component, similar to Jamrozik *et al.* (1997) and Padilha *et al.* (2015), which indicates the ability of TD models and RRM in particular in grasping non-genetic variability among test days.

Table 4: Heritability (%) estimates of legendre polynomial (LP) coefficients for 305-day milk, fat, and protein yields, fat percent, protein percent and electrical conductivity (EC) under random regression model

LP additive coefficient	Trait					
	Milk yield	Fat %	Fat yield	Protein%	Protein yield	EC
a_0	0.934	21.971	1.502	0.725	0.988	0.038
a_1	54.230	5.044	46.359	16.976	50.292	0.605
a_2	2.928	16.056	7.991	2.074	1.347	0.017
a_3	0.404	0.431	0.209	3.118	0.581	0.042

Source: Authors' own elaboration.

Table 5: Permanent environmental component (%) estimates of Legendre polynomial coefficients for 305-day milk, fat and protein yields, fat percent, protein percent and electrical conductivity under random regression model

Perm. envi. coefficient	Trait					
	Milk yield	Fat %	Fat yield	Protein%	Protein yield	EC
pe_0	78.895	52.288	74.405	15.293	75.519	0.020
pe_1	0.821	21.256	0.414	0.226	0.784	0.014
pe_2	13.945	0.560	8.053	7.951	12.052	0.014
pe_3	3.253	7.472	2.394	0.509	6.608	0.015

Source: Authors' own elaboration.

Table 6: Model selection criteria - log likelihood and Akaike information criterion (AIC), likelihood ratio test (LRT) and chi-square statistic (x^2) for repeatability (RPM) and random regression (RRM) models

Model	p	-2Log L	AIC	LRT	x^2 value
RPM	84	83 179.331	83 347.331		
RRM	210	82 337.202	82 757.202	842.129**	153.19

Source: Authors' own elaboration.
Note: **$p < 0.01$.

Table 7: rank correlations among sires for the six traits under repeatability (RPM) and random regression (RRM) models

Trait	Model	Milk yield		Fat %		Fat yield		Protein %		Protein yield		EC[1]	
		RPM	RRM	RPM	RRM	RPM	RRM	RPM	RRM	RPM	RRM	RPM	RRM
Milk yield	RPM	1.00	0.30	0.99**	0.72**	0.99**	0.43*	-0.91**	-0.23	0.99**	0.29	-0.75**	-0.25
	RRM	0.30	1.00	0.31	0.39*	0.31	0.89**	-0.31	-0.23	0.29	0.92**	-0.02	-0.18
Fat %	RPM	0.99**	0.31	1.00	0.72**	0.99**	0.43*	-0.92**	-0.21	0.99**	0.30	-0.74**	-0.23
	RRM	0.72**	0.39*	0.72**	1.00	0.72**	0.66**	-0.57**	0.13	0.71**	0.56**	-0.53**	-0.09
Fat Yield	RPM	0.99**	0.31	0.99**	0.72**	1.00	0.43*	-0.91**	-0.21	0.99**	0.30	-0.74**	-0.23
	RRM	0.43*	0.89**	0.43*	0.66**	0.43*	1.00	-0.37	-0.03	0.41*	0.97**	-0.20	-0.24
Protein %	RPM	-0.91**	-0.31	-0.92**	-0.57**	-0.92**	-0.37	1.00	0.40*	-0.89**	-0.23	0.75**	0.38*
	RRM	-0.23	-0.23	-0.21	0.13	-0.21	-0.03	0.40	1.00	-0.19	0.06	0.33	0.49**
Prot. yield	RPM	0.99**	0.29	0.99**	0.71**	0.99**	0.41*	-0.89**	-0.19	1.00	0.28	-0.74**	-0.23
	RRM	0.29	0.92**	0.30	0.56**	0.30	0.97**	-0.23	0.06	0.28	1.00	-0.05	-0.12
EC	RPM	-0.75**	-0.02	-0.74**	-0.53**	-0.74**	-0.20	0.75**	0.33	-0.74**	-0.05	1.00	0.62**
	RRM	-0.25	-0.18	-0.23	-0.09	-0.23	-0.24	0.38*	0.49**	-0.23	-0.12	0.62**	1.00

Source: Authors' own elaboration.
Notes: a [1] EC: Electrical Conductivity.
b * $p < 0.05$ and ** for $p < 0.01$.

For RPM and RRM models, Spearman's sire rank correlations among the estimated breeding values for yield traits were quite strong except for protein percent (Table 6). Rank correlations between the estimated breeding values from RPM and RRM for individual traits were significantly positive ($p < 0.05$) for all traits except for protein yield which shows that either model is suitable for sire evaluations. However, lower AIC values and significant log likelihood ratio test ($p < 0.05$) in Table 7 show that the RRM with more parameters provides a better fit, as in (Padilha *et al.*, 2015).

4. Conclusions

An organized TD recording programme is essential to obtain data on milk component yields at field level where smallholder farmers have no recording equipment. The genetic correlation structure among traits and sire rankings shows the need for implementing an index-based selection approach to improve the economic traits simultaneously. With a superior fit, the RRM can be recommended for TD animal model evaluations of dairy cattle for the above-described field level scenarios.

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Advances in biotechnologies for improving livestock breeding and feeding

Successful stories on sheep artificial insemination towards promoting the technique in the developing world

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1. Introduction

Reproductive technologies have been applied for many years in advanced intensive livestock farming systems in economically developed countries. However, these technologies can also be very beneficial in other situations (extreme farming conditions, organic farms, developing countries) with a variety of different goals (reduction of cost, simplification, avoidance of hormones use). For these purposes, the Agricultural Research Agency of Sardinia, Italy (Agris) has developed different protocols for artificial insemination (AI) of small ruminants (Colas *et al.*, 1968; Colas *et al.*, 1980).

In 1990 in Sardinia, a novel programme of small ruminant AI began, involving around 400 farms and about 20 000 animals distributed in different Sardinian territories. One of the most important challenges to be addressed was the logistics of rapid transport. The semen collected needed to reach 16–20 farms a day, with an average of 50 animals per farm to be inseminated (~20 percent of the flock size) within a few hours from collection, for 24 days starting at the beginning of the Sardinian breeding season (May–June) (Sanna *et al.*, 1995).

In Sardinia, two different systems are used for sheep production, (i) conventional and (ii) organic. In the paper, we will first discuss the typical method of preparation and distribution of semen. We will then describe the AI protocol used in Sardinia conventional farming with the use of hormones, followed by a discussion of the protocol used in organic farming without the use of hormones (Dattena *et al.*, 2012; Mayorga *et al.*, 2019). Finally, we will provide some conclusions and recommendations.

2. Preparation and distribution of semen

Both farming systems rely on the services of the Genetic Centre, a station where breeding rams are housed and where semen is collected and processed. Each morning from May to June the semen is collected at 8:00 am from donor rams according to the genetic record of the females that will be inseminated. The semen is evaluated, diluted with a commercial medium (Ovixcell IMV) (400×10^6 spermatozoa/dose) and packaged at 15 °C in 0.25 ml straws printed with the ram identification number and the date of collection and preparation. The straws are then placed in a thermos at 15 °C containing a vial of frozen glacial acetic acid (30 ml) which is isolated from the semen by a polystyrene container. The thermos are then placed in a temperature-insulated bag together with two ice packs to keep the temperature at 15 °C. The bags are delivered to cooperating veterinarians at a previously chosen drop-off point along the main north-south road in Sardinia. Delivery occurs between 11:00 am and 2:00 pm. For this step protocol, it has to be underlined that the timely rapid transportation of chilled semen to the farms is challenging (Mara *et al.*, 2005).

Source: Authors' own elaboration.

Figure 1: Technician assisted during artificial insemination

In the case of organic farming, when sheep are detected in oestrous, the farmer will contact the Genetic Centre for the quantity of semen doses needed. Semen will be collected and processed according to the previously described procedure and then kept at 4 °C in a 15 ml Falcon tube with the ram identification number and the time of preparation. Each farmer will come to collect the semen, which is placed in an insulated cooling box at 4 °C for the transport. The semen can then be kept in ordinary home fridge at 4 °C until use (for not more than 24 hours). During daytime the farmer will continue to check the females for heat every 4 hours. Artificial insemination is done 24 hours after oestrus detection. Once completed, the farmer will inform the Genetic Centre regarding the number of doses needed for the next day. Normally this activity will last three or four days depending on the synchronisation grade of the animals. The technique of "ram effect" is used and synchronizes the animals very well. Most of the females are on heat early in the morning. Therefore, experienced farmers may check for heat only two times a day. Strategies such as this are discussed with the technicians of the Genetic Centre and adjusted accordingly. The chilled semen performs very well within the 24 hours from the preparation if kept in the thermos in the fridge at 4 °C.

3. Genetic Centre ram supply

The Sardinia Ram Genetic Centre receives for free every year a number of young rams (6–8 months old) from the farmers participating in the AI campaign across all of Sardinia. The animals chosen are sons of genetically selected dams and sires according to a breeding programme based on milk production. Each farmer donates at least one male lamb born from the AI campaign of the year before. This ram is chosen by the experts of the Genetic Centre according to the goals of the selection programme.

Upon arrival, the rams initially stay in a quarantine station for a health check-up before their acceptance into the centre. Then, the young animal will then be trained for semen collection.

4. Ram evaluation and training

The breeders' association will evaluate the morphology of the rams and their genetic value. Agris technicians will start to train the young rams to use an artificial vagina. This training will last about three months. Only 30 percent of these (Sarda breed) rams will successfully accept the use of the artificial vagina within the first period of training, another 30 percent will learn after one year of training and approximately 30 percent of animals never learn it. The trained animals will be used in the next AI campaign to verify their genetic characteristics.

5. AI conventional farm protocol in Sardinia

The conventional farming protocol for AI is based on two parameters: (i) synchronisation of 16 farms per day; and (ii) hormonal treatment and timing of insemination.

Hormonal treatment starts on day 0 at 8:00 am with insertion of sponges (20 mg Cronolone). At 8:00 am on day 13 sponges are withdrawn and an intramuscular injection of 400 IU of Pregnant Mare Serum Gonadotropin (PMSG) is administered by a veterinarian. Between 12:00 pm and 2:00 pm of day 15 all farms receive the temperature-insulated bag containing a thermo with the semen kept at 15 °C. At 3:00 pm the veterinarian or AI technician performs the insemination (55 h after sponges were removed) with the help of the farm staff as required (Figure 1).

6. AI in organic farming protocol

The organic farming protocol is based on three points: (i) synchronisation of the ewes with the ram effect; (ii) semen technology by using chilled semen at 4 °C; and (iii) farmers trained for the use of ram effect, oestrus detection and AI. This last point is probably the most important and challenging.

Ewe synchronisation in organic farming needs rigorous management of animals and appropriate semen preservation. It consists of a few important steps:

- **Isolation** of females from males for at least 8 weeks prior to start the ram introduction.
- **Introduction of rams** for oestrous synchronization (ram effect).
- **Oestrous detection** with the help of rams wearing an apron, up to 4 times per day (8:00, 12:00, 16:00, 20:00) 17–24 days after introduction of the ram (Figure3).
- **Semen storage** at 4 °C. When semen arrives at the farm in a temperature-controlled box at 4 °C, it needs to be placed in a thermos previously stabilized at 4 °C and placed in the refrigerator. In this way the semen will remain viable for the next 24 hours.
- **Artificial insemination** will be carried out 24 hours after oestrous detection. A few minutes before the insemination, the farmer will remove the thermos from the refrigerator to carry out the AI. The farmer will fill a 0.25 ml straw, load the pistolet and carry out the AI with the help of someone that handles the animals (Figure 2 and Figure 3).

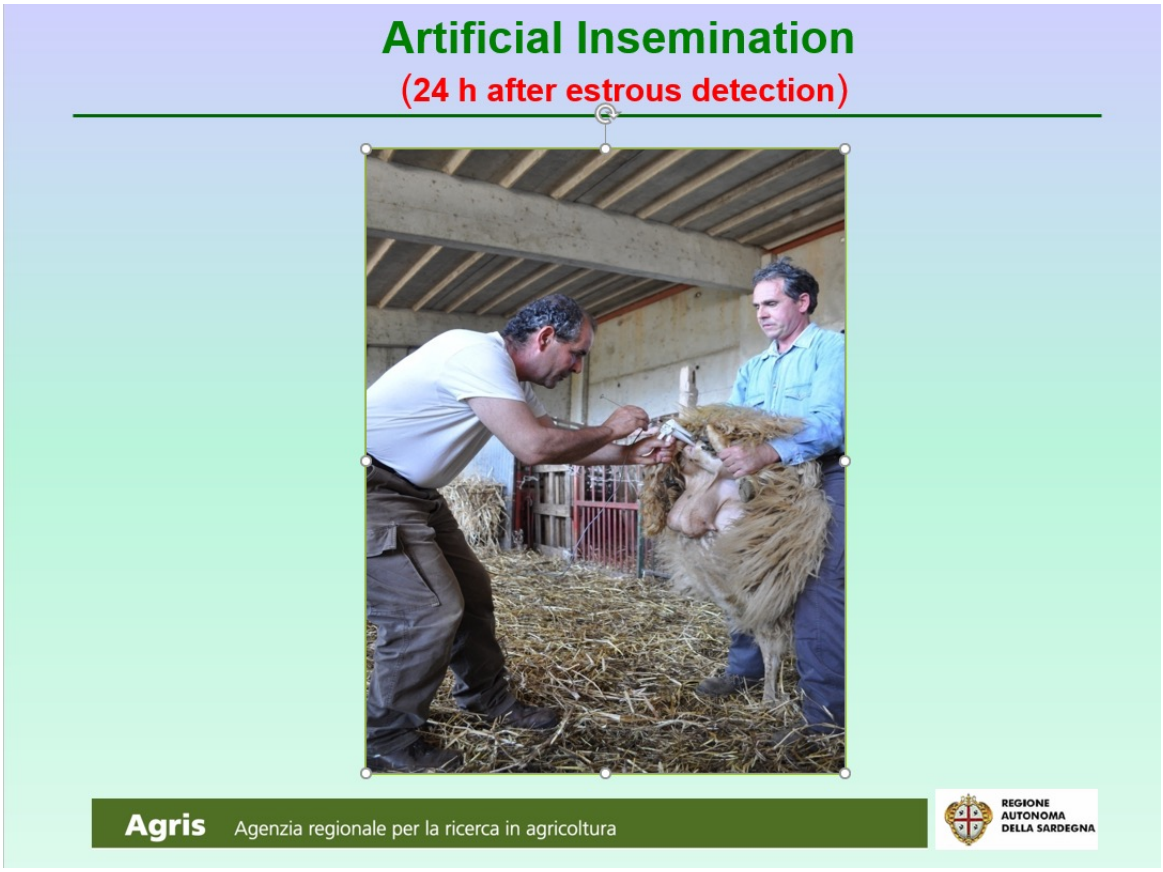
Figure 4 shows results of four years heat synchronisation on a typical farm using the ram effect starting in 2008. The best results in this case were obtained during 2011, the final year shown, presumably because of gradually increasing proficiency through experience gained each year. Indeed, after four years of using the ram effect all animals were synchronised within four days and underwent AI with a 45 percent pregnancy rate, which is relatively good for the Sarda breed.

7. Conclusions

The semen collection, AI and oestrous synchronisation technologies are well established in Agris and many farmers are willing to participate in the programme. The average pregnancy rate in both systems is 45 percent (ranging between 30 percent and 60 percent) with big differences depending on the farm. These techniques are, however, justifiable only in situations where genetic improvement is obtained. Although the pregnancy rate may be low relative to natural mating, progress towards the objective to improve the genetic merit of a small population of sheep in marginal and isolated territory can outweigh the disadvantages of a lower pregnancy rate. In our opinion, both methods (those applied on conventional and organic systems) and their logistics can be applied in many different situations and conditions, including in developing countries. The biggest challenge

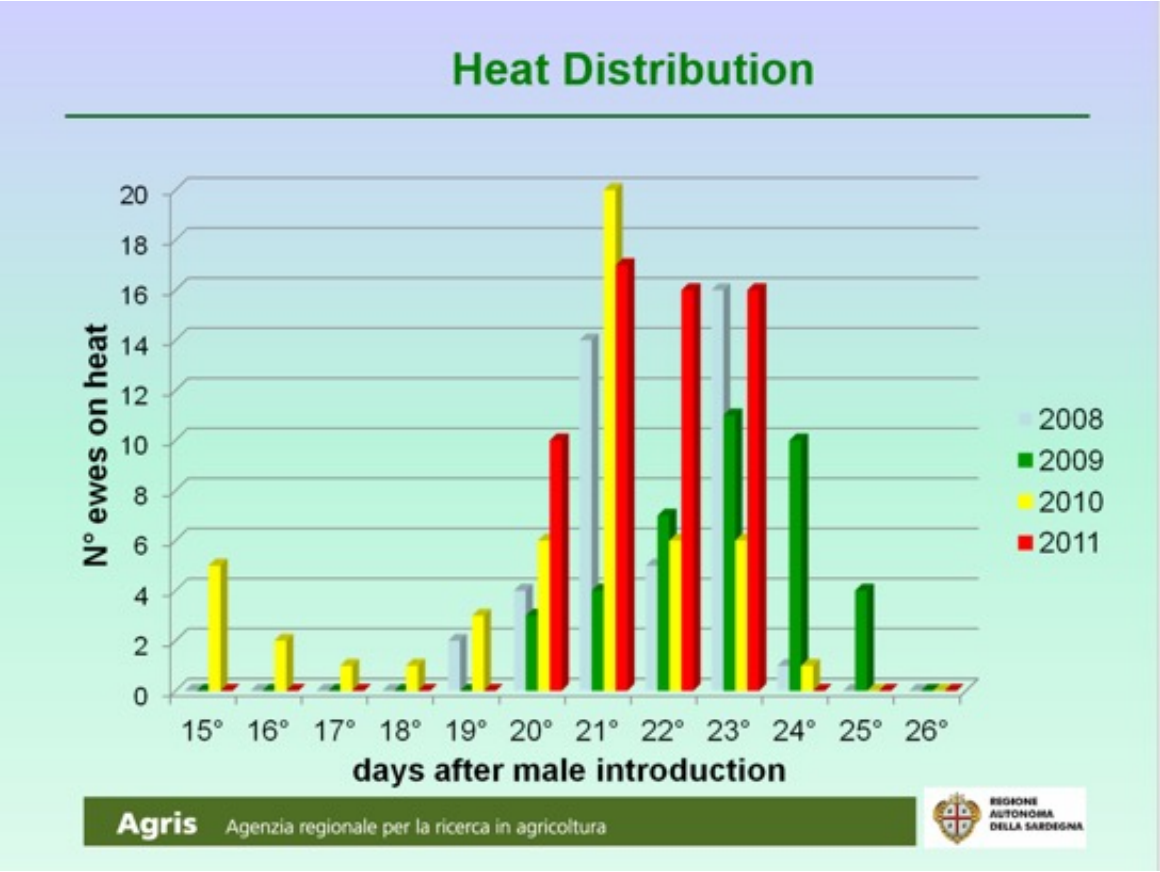
Source: Authors' own elaboration.

Figure 2: Farmer filling the straw



Source: Authors' own elaboration.

Figure 3: Performing artificial insemination



Source: Authors' own elaboration.

Figure 4: Four-year heat distribution in a representative organic farm

is the continuous updating and investment in training of farmers, technicians and veterinarians to ensure continuity and continual improvement. This challenge can be reached by strengthening connection and collaboration and sharing the costs among farmers, veterinary, animal science and agriculture schools and national or regional researcher centres.

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Using molecular and near nuclear technologies to understand embryo-maternal interactions; Can we identify an early pregnancy biomarker in cattle?

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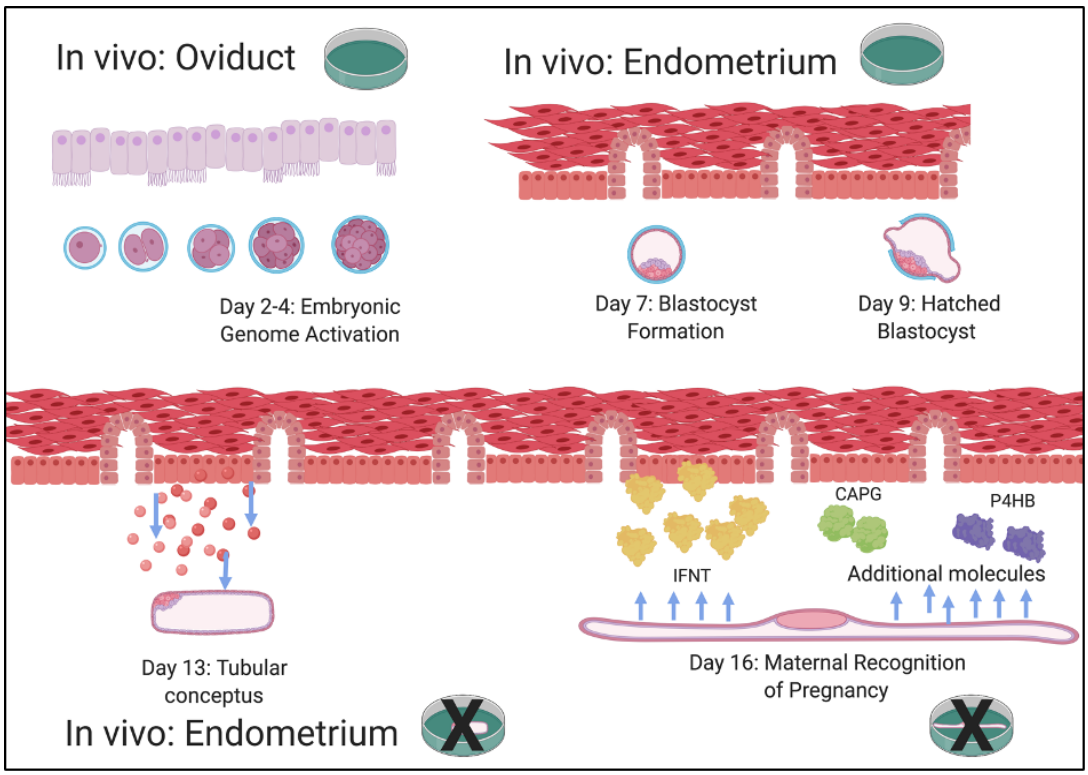
Abstract

In cattle, the majority of pregnancy loss occurs in the first 4 weeks of pregnancy, i.e. in the pre- and peri-implantation periods. Recent studies have determined a major wave of pregnancy loss occurs between days 8 and 27 in dairy cows. During this time, the conceptus (embryo and associated extra-embryonic membranes) undergoes pivotal developmental stages including elongation of the conceptus, maternal recognition of pregnancy, maintenance of the corpus luteum, and implantation. These are all time-points during which pregnancy loss can occur. Establishing a robust and reliable pregnancy biomarker during this period that is useable in a variety of production systems would help sustainably improve food production. One potential biomarker is the pregnancy recognition signal Interferon tau (IFNT). Measuring the response of the maternal environment to IFNT can prove difficult, however. IFNT triggers a classical IFN response which can be detected in blood. However, this is similar to that triggered by any type 1 interferon, i.e. it's not specific to pregnancy. More recent efforts have therefore focussed on other conceptus-derived proteins that are *de novo* synthesised by the conceptus as well as components of extracellular vesicles produced by the conceptus. By understanding the fundamental biology of early pregnancy, we will be able to develop novel pregnancy biomarkers that have the potential to be used in a variety of production systems to sustainably enhance food production.

1. Introduction

Livestock (including both beef and dairy farming) contribute to 40 percent of the value of agricultural output worldwide. Livestock farming also supports the economic livelihood and food security of ~1.3 billion people. Alongside external pressures including adverse effects of climate change and increased competition for land use and fresh water, agriculture and food security are of critical importance if we are to sustainably meet the food demands of an increasing population. Establishing and maintaining successful pregnancies is essential for the economic viability of farmers both in terms of lactation (milk and associated products) as well as animals for meat consumption. A major factor affecting the efficiency and sustainability of both dairy and beef farming is poor reproductive outcomes associated with embryo mortality.

In cattle, the majority of embryo loss occurs in the pre- and peri-implantation period of pregnancy, i.e. the first 4 weeks of pregnancy. Many factors can contribute to this loss, including genetic abnormalities in the embryo (VanRaden and Miller, 2006), errors in fertilisation, aberrant embryonic development, nutritional status, circulating concentrations of progesterone, as well as negative energy balance associated with lactation and/or heat stress (Butler, 2003). In high-producing dairy cows, up to 50 percent of pregnancy loss occurs in a first "wave" that occurs in the first 7 days post-conception (Wiltbank *et al.*, 2016), with relatively little fertilization failure (<10 percent) or late embryonic loss (~20 percent) (Diskin and Morris, 2008). A second major pivotal time of pregnancy loss occurs between days 8 and 27 and is responsible for 30 percent of pregnancy loss in dairy cows. This is the time when the conceptus undergoes key developmental checkpoints including elongation, maternal recognition of pregnancy, and implantation (Figure 1). This loss can be attributed to dysfunction in embryo development as well as defective molecular communication between the



Source: Authors' own elaboration.

Figure 1: Following successful fertilisation, the embryo enters the uterus on day 4; *in vitro* production of embryos up to the blastocyst stage of development is possible; the blastocyst hatches from the zona pellucida and the trophoblast undergoes a period of proliferation to produce an elongated conceptus producing signalling molecules including IFNT (maternal recognition of pregnancy signal); this is the time period where up to 30 percent of pregnancy loss can occur

embryo and the maternal uterine environment required for successful implantation (Diskin and Morris, 2008; Wiltbank *et al.*, 2016). Being able to determine if pregnancy has been successful past this hurdle of the second wave of pregnancy loss would be of great benefit to farmers in a variety of production systems. In this mini-review we bring together information on the process of molecular communication between the conceptus and endometrium, and how this can be used to form the basis of a pregnancy diagnostic to improve food production in a variety of systems.

2. Events in early pregnancy

Following successful fertilisation and several rounds of cell divisions where the embryonic genome is activated in the oviduct, the bovine embryo (at the morula stage) enters the uterus on approximately day 4 post-fertilisation (Valadão, Moreira da Silva and Moreira da Silva, 2019). The embryo continues to undergo cell division until reaching around 100 cells at the blastocyst stage, which hatches from the zona pellucida (Valadão, Moreira da Silva and Moreira da Silva, 2019) on day 8–9 (Van Soom *et al.*, 1997). These processes do not require the maternal uterine environment, as evidenced by *in vitro* produced blastocyst transfer to synchronised heifers resulting in successful pregnancy (Brackett *et al.*, 1982). While there is a response of the oviduct (Maillo *et al.*, 2015) and the endometrium (Sponchiado *et al.*, 2017) to these stages of development, these responses seem to be localised in nature and are unlikely to yield a systemically detectable signal of successful pregnancy. Moreover, given the second wave of embryo loss occurs from days 8 to 27, this time interval would be a more appropriate focus for systemically detectable pregnancy signals given the greater likelihood of an established pregnancy being maintained.

The hatched bovine blastocyst undergoes a period of rapid elongation from days 13 to 16, increasing over 1000-fold in size to form an ovoid, then tubular, and finally a filamentous structure (Maddox-Hyttel *et al.*, 2003). By day 16 the bovine conceptus (embryo and extra-embryonic membranes) usually occupies the full length of the ipsilateral horn (Kastelic *et al.*, 1988). By days 19 to 20, the bovine conceptus usually spans both uterine horns and has implanted/adhered to the endometrium by day 21. This process is entirely reliant on secretions from the luminal and glandular epithelium of the endometrium – collectively termed uterine luminal fluid (ULF). This process is entirely maternally derived as is evidenced by the uterine gland knockout ewe model whereby ablation of uterine glands and epithelium results in the inability of the uterus to support elongation post transfer of morphologically normal embryos (Allison Gray *et al.*, 2000; Gray *et al.*, 2001; Filant and Spencer, 2013). Moreover, attempts to recapitulate this process *in vitro* have been relatively unsuccessful (Vajta, Alexopoulos and Callesen,

2004; Machado *et al.,* 2012). We do know that, at these post-hatching, elongated stages of development that the conceptus alters the uterine endometrial transcriptome – particularly during the processes of establishing uterine receptivity to implantation and maternal recognition of pregnancy recognition (Bauersachs *et al.,* 2006; Forde *et al.,* 2011; Forde *et al.,* 2012; Sánchez *et al.,* 2019b). These are more systemic responses and are likely to be a more fruitful option to investigate for a signal of pregnancy success.

3. Maternal recognition of pregnancy in cattle

Maternal recognition of pregnancy (MRP) is accomplished by the molecular crosstalk between the embryo and maternal environment to block luteolysis of the corpus luteum (CL) and maintain its production of progesterone (P4) required to sustain pregnancy. If the embryo-derived factor (pregnancy recognition signal) is not present in sufficient quantities (lack of conceptus or conceptus not producing sufficient signal), the CL will undergo luteolysis, P4 concentrations in circulation will drop accordingly, and the estrous/menstrual cycle will continue (Bazer, 1992).

Interferon tau (IFNT) is the pregnancy recognition signal in all domestic ruminant species, including cattle and sheep. First identified in the 1970's (Wilson, Lewis and Bazer, 1975; Lewis *et al.,* 1979), IFNT is a ruminant-specific type I interferon cytokine, in the same family as IFN gamma and omega.

By day 16 of pregnancy the trophoblast cells of a competent, sufficiently elongated filamentous conceptus, will have secreted sufficient bovine IFNT (bIFNT) to stimulate MRP (Helmer *et al.,* 1989; Thatcher *et al.,* 1989). While bIFNT is secreted from the blastocyst stage (Hernandez-Ledezma *et al.,* 1993), with bIFNT messenger RNA (mRNA) detectable in the 16-cell IVF-produced bovine blastocyst (Yao *et al.,* 2009), it is only during the elongation and pregnancy recognition process that it is produced in appreciable quantities. Moreover, the quantity of IFNT secreted by the conceptus has been linked to conceptus length, with longer conceptuses producing greater quantities of IFNT and being more likely to maintain P4 production by the CL, and subsequently result in successful pregnancy (Matsuyama *et al.,* 2012). The transcriptional changes within the endometrium induced in response to IFNT and/or pregnancy have been well characterised in many studies (Bauersachs *et al.,* 2012; Mathew *et al.*, 2019; Sánchez *et al.,* 2019a). Principally, IFNT binds the IFNAR receptor expressed in the cells of the endometrium and triggers the JAK-STAT1 and TYK-STAT2 pathways, resulting in activation of the ISRE gene promotor and the transcription of IFN-stimulated genes (ISGs)- including *ISG15, MX1* and *MX2* (Bauersachs *et al.,* 2012). Interestingly, the expression of these genes is induced by all type I IFNs and are not unique to IFNT (Michalska *et al.,* 2018) rendering it difficult to use classical ISGs as the basis of a pregnancy test as they can be triggered by other type I IFN sources such as viruses. Alongside classical ISGs, non-classical ISGs (i.e. not induced by other type I IFNs) induced by IFNT have also been characterised in cattle (Bauersachs *et al.,* 2006; Forde *et al.,* 2011) and sheep (Chen *et al.,* 2006). Attempts have been made to detect these ISGs in circulation but the results seem to be somewhat muddied in cows compared to heifers (Green *et al.,* 2010; Okumu *et al.,* 2011; Rocha *et al.,* 2020).

Not only does the female tract respond to the mere presence of an embryo/conceptus but it also responds to the developmental potential of the conceptus. This biosensor capability, specifically of the endometrium, was first identified as a modified response of the endometrium to the pregnancy recognition process when embryos with different developmental competencies were present (Bauersachs *et al.*, 2009; Mansouri-Attia *et al.,* 2009). Differences in foetal morphology (Hue *et al.*, 2019), metabolic differences in the pre-implantation embryo (Khurana and Niemann, 2000), developmental differences in offspring, and lower rates of pregnancy success from *in vitro* produced blastocysts (Farin and Farin, 1995) have previously been extensively reviewed (Farin, Crosier and Farin, 2001). However, the pregnancy recognition response was modified depending on the source of the embryo, i.e. *in vivo*, *in vitro*, or clones although our understanding of what is driving this biosensor response is still unclear. However, furthering our understanding of this process could lead to a scenario whereby we could not only detect likelihood of pregnancy success but also the developmental potential of the pregnancy.

4. Additional molecular signals between the conceptus and endometrium

Along with the discovery that IFNT was the pregnancy recognition signal in cattle (Lewis *et al.,* 1979), studies have detected additional proteins that were produced by the conceptus and could potentially interact with the endometrium and more systemically. These proteins can be classically secreted proteins (have a secretory consensus sequence) or non-classically secreted proteins. The bovine conceptus has also been demonstrated to secrete prostaglandins (Forde *et al.,* 2011), and proteins into the uterine luminal fluid (ULF) on day 16 (Forde *et al.,* 2015) of pregnancy which coincides with pregnancy recognition in cattle.

A combination of *in vivo* and *in vitro* methods (Forde *et al.,* 2015) used liquid chromatography and mass spectroscopy to identify 30 proteins unique to the ULF of pregnant heifers which were also secreted by the conceptus *in vitro.* The 30 proteins identified included: HSPE1, YWHAG, PSMC4, ACO2, ARPC5L, ACTN1, GLB1, PRKAR2A,

CKMT1, EEF2, LGALS3, HNRNPA1, HNRNPF, HNRNPA2B1, IDH2, KRT75, CAPG, MSN, NUTF2, PGM2, GPLD1, PSMA4, PSMB5, P4HB, GDI1, CSTB, HSPA8, SERPINA3, TXN and TKT (Forde *et al.,* 2015). They also identified IFNT as conceptus-derived, the well characterised bovine pregnancy recognition signal. IFNT was identified in ULF on days 16 and 19, but not on days 10 or 13. Many of the other 30 proteins identified were present in the ULF at all four time points, indicating these proteins may have roles in conceptus-endometrial interactions throughout early pregnancy.

Extracellular vesicles (EVs) are membrane-bound lipid bilayer vesicles that are released from the plasma membrane into the extracellular space and have been identified in many biological fluids, including plasma, saliva, urine, and ULF (Burns *et al.,* 2014). The three main subsets of EVs, namely apoptotic vesicles, microvesicles (MVs) and exosomes, are distinguished by their diameter. Apoptotic bodies are 1 000 – 5 000 nm diameter, MVs range from 20 – 1 000nm and exosomes 50 – 100nm (van der Pol *et al.,* 2012). Due to the different subtypes of EVs having overlapping size ranges, it is now recommended to classify EVs as either small (sEV), medium (mEV) or large (lEV) if classifying by size. sEVs are < 100 nm, mEVs 100 – 200 nm and lEVs > 200 nm (Théry *et al.*, 2018). It is also possible to characterise EVs by protein content, surface markers and density (Théry *et al.*, 2018). EVs of all sizes have been shown to contain a variety of components, including: functional proteins (Gidlöf *et al.,* 2019), lipids (Chen *et al.,* 2019), DNA (Vagner *et al.,* 2018), mRNAs (Valadi *et al.,* 2007), mircro RNAs (miRNAs) (Valadi *et al.,* 2007), and long non-coding RNAs (lncRNAs) (Hinger *et al.,* 2018). Their contents are of particular importance in cell-cell communication because their lipid bilayer protects their cargo in the extra-cellular space. The miRNAs have been shown to be differentially packaged, as they are enriched relative to the parent cell (Hessvik *et al.,* 2012), which is thought to be modulated by nuclear ribonucleoprotein A2B1 (Villarroya-Beltri *et al.,* 2013) and KRAS (Cha *et al.*, 2015). Exosomal mRNAs also show enrichment for specific mRNAs with 3'UTR fragments (Batagov and Kurochkin, 2013). Ubiquitinated proteins are also enriched in exosomes, with ubiquitination thought to target proteins for packaging into exosomes (Cheng and Schorey, 2016). After docking and being internalised, EV contents are released into the cytoplasm of their target cells, following which mRNAs can undergo translation (Valadi *et al.,* 2007), miRNAs can regulate gene expression on a post-transcriptional level (Montecalvo *et al.,* 2012) and functional proteins can contribute to cellular processes.

Certain EVs have been isolated from the conditioned media produced by bovine (Mellisho *et al.,* 2017) *in vitro* produced blastocysts. Bovine day 7 to 9 blastocyst-derived EVs ranged from 30 – 385 nm, characteristic of both exosomes and MVs. They contained CD63 and CD9, common exosomal markers (Mellisho *et al.,* 2017). Other studies identified a miRNA signature that differentiated between embryos of different developmental success (Melo-Baez *et al.*, 2020). A study on infusion of labelled EVs in sheep ULF demonstrated trophoblast-derived EVs were specifically taken up by endometrial epithelial cells and the trophectoderm, indicating that endometrial derived EVs contribute to both conceptus and endometrial development (Burns, Brooks and Spencer, 2016) and also identified an mRNA and protein signature of conceptus-derived EVs. This study demonstrates in a ruminant species that the ULF contains EVs from the endometrium and conceptus, which are taken up by both the trophectoderm and endometrial epithelium. A second study investigated the content of EVs in the ovine pregnant ULF and their impact upon primary endometrial epithelial cells. They identified 172 exosomal proteins including some of those previously determined in bovine conceptus-conditioned medium (CAPG, AKR1B1, BLC2L15, CA2, IDH2, EEF2, MSN and EZR) (Nakamura *et al.,* 2016). These studies demonstrate that EV components and their downstream effectors have potential to be markers of successful pregnancy.

5. Novel models using nuclear and near nuclear technologies to identify pregnancy biomarkers

The crosstalk that occurs between embryo and endometrium is clearly complex and bi-directional, making it particularly difficult to determine the source of the signals and the specific sequence in which they occur. Using *in vivo* animal models to study pregnancy recognition signalling is not ideal, as IFNT is a potent cytokine that triggers a type I immune response both within the uterus and within the peripheral systems (Shirasuna *et al.,* 2012). The IFN response is likely to cloud the effects of other conceptus-derived factors within an *in vivo* ruminant model. It is also difficult to determine the source of the factors when comparing cyclic and pregnant animals, as the ULF and endometrium change in response to pregnancy, and therefore it cannot be assumed that those changes identified in the pregnant group are embryo-derived or altered due to embryo-derived factors.

Some strategies to overcome this involve using novel *in vitro* approaches. Static culture of cells in dishes or wells has been used extensively to study the effects of IFNT on different cell types (Asselin, Lacroix and Fortier, 1997; Guzeloglu, Michel and Thatcher, 2004; Mathew *et al.,* 2019), however, these are limited in that the cells are co-cultured with purified IFNT in a single dose whereas, *in vivo*, the endometrium is exposed to increasing concentrations over the pregnancy recognition period. One way to better capitulate this process is to use a

microfluidics approach such as that utilised by De Bem *et al.* (2021) which would allow the exposure of cells to increasing doses of IFNT *in vitro*, similar to what occurs *in vivo*. There are also methods which enable the culture of endometrial spheroids formed from endometrial cells (Yamauchi *et al.*, 2003). These spheroids can be composed of both stromal and epithelial cells and have been shown to form gland-like structures and interact with trophoblast cells (Buck *et al.*, 2015). These 3D co-culture systems allow the cells to self-assemble in endometria-like structures and can be cultured with treatments to analyse the effects in an endometrial-like spheroid. In addition, after culturing *in vivo*-derived conceptuses for a short term *in vitro* (Forde *et al.*, 2015) the secreted factors can then be compared to those unique to pregnant ULF and can be hypothesised to be conceptus derived. However, there are limitations, as some of these secreted factors may be a consequence of the culture system and not reflect the biology of the conceptus during early pregnancy.

The use of radioisotopes to measure concentrations of progesterone in blood via radioimmunoassay has been standard in research laboratories for a long time now. Indeed, commercial measurement of in-line milk P4 (on the biological basis that high P4 after the time when pregnancy recognition has occurred would reflect likelihood of a good pregnancy outcome) currently routine practice by some large, high-producing commercial farms particularly in the US (Pennington, Spahr and Lodge, 1976). Regular monitoring of P4 does present some practical issues, however, regardless of whether blood or milk is used. The collection and use of blood samples can be complicated. Measuring milk P4 limits the testing programme to only those cows that are undergoing lactation. While inline scanning of milk for P4 is a possibility under some circumstances, it requires costly machinery as well as a skilled operator and is therefore not accessible to all farmers and production systems.

As mentioned above, it is difficult to assess the conceptus during the pregnancy recognition window *in vitro*. One approach to determine the health of the embryo/conceptus would be to determine if the conceptus-produced biomarkers are detectable in blood. In an IAEA-funded project, we used an approach involving stable isotope labelling of amino acids in cell culture (SILAC). We used culture media-depleted of the amino acids which we then supplemented with heavy isotope labelled amino acids. Culturing the conceptus in this media allows us to undertake mass spectrometry analysis to determine which proteins are *de novo* synthesised by the conceptus, i.e. we can differentiate them based on containing the heavy or light isotopes of the protein of interest. These proteins could therefore be markers of conceptus developmental competency. We recently identified > 800 proteins that were *de novo* synthesised using this method, and are also components of EVs isolated from the ULF of pregnant animals (Malo Estepa *et al.*, 2020). Work is now underway to determine if any of these or indeed downstream components of EVs are detectable in serum or a similarly accessible sample material.

6. Conclusions

Nuclear and near-nuclear technologies have played an important role in furthering our understanding of the fundamentals required for early pregnancy success in cattle. More recently, near-nuclear and molecular techniques have allowed us to identify how the endometrium responds to the embryo and how that is modified depending on the developmental competency of the conceptus. We have now begun to understand both the traditional protein-ligand interactions that occur between the conceptus and the endometrium during the peri-implantation period of pregnancy, as well as the potential of EV-mediated conceptus-maternal interactions. These studies are leading toward a more fundamental understanding of the biology of early pregnancy to develop novel candidates of biologically based biomarkers of early pregnancy, and have the potential to be used in a variety of production systems to sustainably enhance food production.

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Using genetics to combat global warming and improve heat tolerance in dairy cows

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Abstract

Between 2001 and 2020, the average global surface temperature was 1.09 °C higher than in the period between 1850 and 1900. This "global warming" is expected to result in increased periods of drought and reduced production levels in dairy cattle in many parts of the world. Dairy cows are emitters of methane, a potent greenhouse gas, in addition to being especially sensitive to high temperatures because of the thermogenic effect of lactation. As a result, breeding for reduced greenhouse gas emissions (especially methane) and improving the ability of cows to tolerate heat have become important traits for consideration in breeding programmes. Here we focus on practical solutions for generating breeding values using data that are either abundantly available in many dairy industries already (through data from national recording programmes), or only available in sparse amounts (from research herds). We use the Australian breeding values of heat tolerance and methane emissions as examples in addition to discussing future options for further development facilitated by genomic selection.

Keywords

genomic selection, global warming, heat tolerance, methane emissions, dairy

1. Introduction

Reducing the dairy industry's impact on climate change and adapting to the consequences of climate change are essential for the future success of dairy industries world-wide. Between 2001 and 2020, the global surface temperature was 1.09 °C higher than in the period between 1850 and 1900 (www.ipcc.ch/report/ar6/wg1/downloads/report/IPCC_AR6_WGI_SPM.pdf). This warming of the Earth is expected to result in increased periods of drought and reduced production levels in Australia (BOM, 2015). Greenhouse gases (GHG), including carbon dioxide (CO_2), methane and nitrous oxide, account for 81 percent, 10 percent and 7 percent of the global GHG inventory, respectively (http://epa.gov) and have dramatically increased, especially from the 1950s (www.climate.gov). Many countries have pledged to apply policies to reduce GHG emissions and subsequently reduce global warming.

Enteric methane emission is a major contributor to GHG emissions, with a global warming potential approximately 28 times that of CO_2. (Gerber *et al.*, 2013). Out of the 20 percent of the global livestock sector's GHG emissions attributed to the dairy cattle industry, approximately half of the GHG emissions are caused by enteric methane emissions (Gerber *et al.*, 2013). In addition to contributing to global warming through methane emissions, dairy cattle are also sensitive to the rising temperatures resulting from

global warming. Hence, reducing methane emissions is an important goal for the dairy industry.

While populations of farm animals in their native country tend to be well adapted to their production environment, selection that focused on production traits may have an unintended negative impact on other traits. In dairy cattle, unfavourable genetic correlations between heat tolerance and production traits have been reported (Nguyen *et al.*, 2016), hence selection for increased production may have led to decreased heat tolerance. A cow can maintain a body temperature of 38.4 °C to 39.1 °C when she is in the thermal neutral zone, with temperatures between 16 °C to 25 °C (Yousef, 1985). Heat tolerance is of particular interest in the Australian largely pasture based dairy production system, where the number of days per year a cow is expected to be exposed to temperatures outside the thermo-neutral range is between 60 (in Tasmania) and 300 (in Queensland) days per year (Nguyen *et al.*, 2016).

Fortunately, it is now possible to use genomic selection to reduce the effects of global warming (i.e. through selecting for heat tolerance) and to reduce the contribution of cows to global warming (i.e. through selecting for reduced emissions). Genomic selection uses animals that have both genotypes and phenotypes to develop genomic prediction equations. Subsequently, the genomic prediction equations are used to predict genomic estimated breeding values for individuals that have genotypes, but no phenotypes of their own (Goddard, Hayes and Meuwissen, 2010). One of the most notable outcomes of genomic selection in dairy cattle has been essentially eliminating the "waiting-period" of progeny-testing. Instead of waiting the 6 years from birth until a reliable estimated breeding value (EBV) is available, breeders are now able to have reliable EBVs of bulls at birth. This change has led to much greater rates of genetic progress per year compared to that in the pre genomic era (García-Ruiz *et al.*, 2016). Additionally, genomic selection also offers countless opportunities for increasing the number of traits that are under selection, especially when matched with reference populations where genotyped individuals have measurements on these new traits. Over the last decade genomic selection has revolutionised selection in plants and animals universally and with extraordinary effect, especially for traits that are either scarcely available (such as data on individual cow methane emissions) or for traits that are sex-linked or only measurable later in life, which covers many traits of importance in dairy cattle breeding.

In this paper we present the role of genetics to combat the effects of climate change associated with dairy production, covering genetic selection approaches being applied in Australia, including a proactive solution (reducing emissions) and a reactive solution (breeding for heat tolerance).

2. Selecting for reduced methane emissions

There are two groups of strategies that can be used to mitigate methane emissions, and these include those that can be implemented immediately, such as dietary interventions, and longer-term solutions, such as genetic selection. Genetic selection could offer a cumulative and permanent solution by reducing methane emissions through either selecting for traits that reduce emissions, or by identifying cows that are low emitters.

Selecting for traits associated with low emissions

Genetic evaluations in many countries provide EBVs on many economic traits to farmers. These EBVs that are currently available to farmers either have a favourable, or unfavourable effect on methane emissions. For example, genetically improving milk yield traits has led to a dilution effect on methane emissions per litre of milk produced. Milk yield in the United States of America has increased to around 10 000 kg in 2015, corresponding to a four-fold increase since 1945 (Britt *et al.*, 2018). Cole *et al.* (2020) estimated that about 50 percent of this gain was due to genetic improvement. As a result of this, the carbon footprint per billion litres of milk produced in the United States of America in 2007 was only 37 percent of that in 1944.

Improving fertility leads to a reduction in the number of replacement heifers needed, and consequently reduces emissions (Garnsworthy, 2004; Bell *et al.*, 2013). Improving longevity and feed efficiency can reduce emissions in a similar manner (Richardson *et al.*, 2021a). Richardson *et al.* (2021a) calculated the CO2-eq/cow/year effect of a unit increase in each of the traits for which EBVs are calculated for Australian dairy cattle. As these coefficients were calculated to be independent of each other, they can be used as weights to form a GHG sub-index and used to identify animals that will be lower emitters of methane (Richardson *et al.*, 2021a).

Selecting for reducing methane directly

Although a GHG index that includes weights applied to EBVs that are already available is a fairly easy way to achieve greater reductions in methane intensity, the next logical step is to measure methane emissions, calculate EBVs and implement direct selection for reducing methane emission. Genomic selection has been an enabling technology in this space, maximizing the investment made in collecting high value phenotypes, such as individual cow records on emissions. There are several methods to measure per cow methane emissions, including enclosed respiration chambers, the sulphur hexafloride tracer technique (SF6), handheld laser methane detection, automated head chambers (e.g. Greenfeed system) and sensors in automated milking systems. Using data collected from these techniques, genetic variation in methane

emissions of cattle has been estimated (de Haas *et al.*, 2017; Garnsworthy *et al.*, 2019; Richardson *et al.*, 2021b).

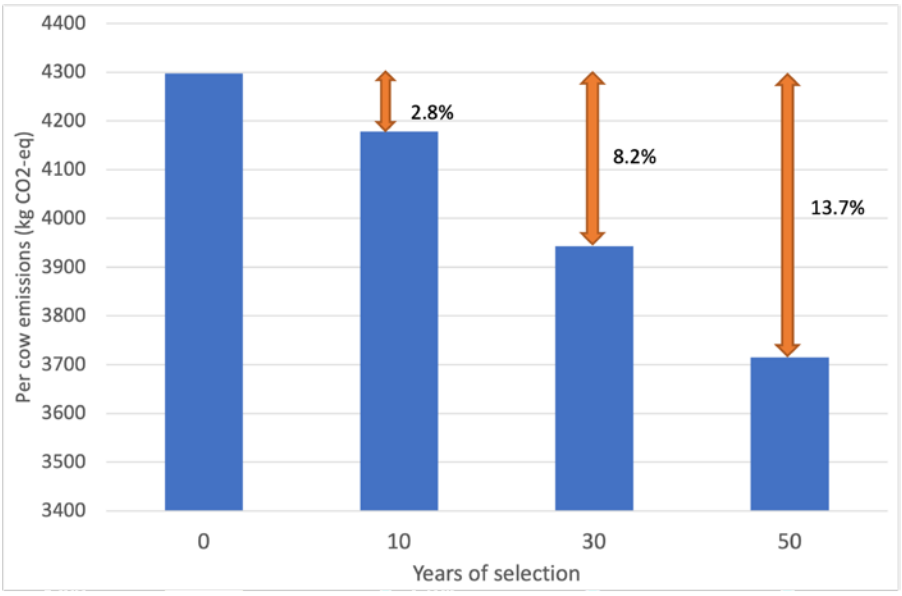
Several studies have investigated definitions of appropriate breeding objectives for inclusion in breeding indices (Breider, Wall and Garnsworthy, 2019; Manzanilla-Pech *et al.*, 2021). An important consideration is that the breeding objective and selection index traits may not be the same. While the objective is to improve methane intensity, the selection criterion could be other traits, such as methane production and milk yield traits. Several studies are now favouring a methane trait that is corrected for milk production traits (Manzanilla-Pech *et al.*, 2021; Richardson *et al.*, 2021a), as this should lead to selecting for low emitters in a way that is independent of milk production.

Genomic prediction requires a large dataset with phenotypic and genomic information to accurately predict an animal's genetic potential. Currently, the size of national datasets for methane are too small for routine genetic evaluations, especially since the prediction equations are generally based on a female driven reference population from a few research farms. Gonzalez-Recio, Coffey and Pryce (2014) suggested that for a moderately heritable trait (e.g. 0.2) in a female driven reference population, approximately 5 000 individual animal records would be needed to reach a reliability high enough to be accepted for implementation ($r = 0.25$). Currently obtaining data of adequate quantity is challenging, therefore, international collaboration is underway to greatly increase the number of animals with recorded methane phenotypes. The Efficient Dairy Genome Project is a large-scale international initiative aimed to increase feed efficiency and decrease methane emissions in dairy cattle through the utilization of genomics (Efficient Dairy Genome Project, 2016). This shared resource includes methane and dry matter intake data from six countries (Australia, Canada, United States of America, Switzerland, United Kingdom of Great Britain and Northern Ireland, and Denmark) and aims to facilitate the sharing of many 1000s of data records.

Even with international collaboration, the reliability of EBVs for a methane trait is going to remain low, so the use of predictor traits may also be useful. Based on recent research the most promising of the common predictor traits seem to be volatile fatty acids (VFA), rumen pH and measurements of mid-infrared spectroscopy (MIR) of milk. Williams *et al.* (2019) have shown that VFA proportions in ruminal4288. fluid can be used to predict methane yield and they have suggested that their approach is among the cheapest methods for estimating methane yield of dairy cows. Rumen pH on the other hand has a strong biological connection to methane, as the process whereby methanogens use excess hydrogen in the rumen to generate methane also drives feed fermentation, with lower rumen pH being associated with reduced ruminal methane production (Van Kessel and Russell, 1996). One option is to increase concentrates fed as a way to inhibit methanogenesis (Van Kessel and Russell, 1996). However, there may also be genetic variation between animals in methanogenesis. As such, the heritable part of the rumen metagenome may also be useful to identify cows that are lower emitters of methane (Delgado *et al.*, 2019; Wallace *et al.*, 2019).

Methane as part of the breeding objective

Multi-trait selection indices that have enabled breeding goals to be expanded include many traits simultaneously (Hazel, 1943). A selection index starts with the identification



Source: Authors' own elaboration.

Figure 1: Response to selection in per cow emissions predicted after 10, 30 and 50 years of selection on an expanded version of the Balanced Performance Index (BPI) that includes carbon at a price of USD 250/tonne; adapted from Richardson *et al.* (2021c) (submitted paper)

of the breeding goal, which is often net farm profit. The breeding goal is calculated as the sum of the EBVs for each trait multiplied by its economic value. Therefore, most indexes used in animal breeding are constructed with a focus on how traits are expected to impact on profitability, although there is growing interest in expanding selection indexes to encompass animal welfare and environmental outcomes (Nielsen, Christensen and Groen, 2005; Boichard and Brochard, 2012) in addition to profitability. With this in mind, Richardson *et al.* (2022) have proposed expanding the Australian Balanced Performance Index (BPI, the economic index) to include a greenhouse gas index with carbon valued at 3 different prices. The greenhouse gas index included coefficients applied to existing EBVs in addition to a residual methane trait. With a value of carbon of USD 250/tonne and assuming conservative and achievable levels of uptake of an adapted version of the BPI, it is estimated that after 30 years of selection, a reduction of gross emissions of 8.2 percent is achievable. As genetic progress in milk production traits is happening at the same time, the reduction in emissions intensity is estimated to be 21 percent (Richardson *et al.*, 2022). Very similar results (24 percent reduction in emissions intensity) have been modelled in the Netherlands using a different approach (de Haas *et al.*, 2021).

Maintaining the social license to operate is extremely important for dairy farmers and breeders, and is becoming more challenging as volumes of information, whether true or false, are easily accessible to consumers. By taking the steps to introduce an environmental-based index, stakeholders can show consumers that environmental stability is also an important focus of the dairy industry.

3. Selecting for heat tolerance

Climate change has already occurred, and further changes are seemingly inevitable, leading to further detrimental effects on grazing ruminants (Gosnell, Charnley and Stanley, 2020), one of which is heat stress. Heat stress causes behavioural, physiological and cellular adaptations that aim to reduce the cow's body temperature (Dunshea *et al.*, 2013). Examples of these changes are a reduction in feed intake and milk production, an increase in respiration rate, heart rate, panting, sweating, changes in coat colour, thickness and length, and behaviours such as seeking shade. How individuals adapt to heat stress may be influenced by nutrition and management, but also has genetic variance that can be captured by genomic selection for improved heat tolerance (Nguyen *et al.*, 2016).

Measuring heat tolerance

Several methods have been developed to obtain heat tolerance phenotypes that can be used for genetic selection. A common measure of heat tolerance is the change in animal performance with the change in bioclimatic indices. For example, the temperature-humidity index (THI) calculated using both temperature and humidity is one important index. Body temperature, respiration rate, heart rate, sweating rate, reduction in intake or milk yield and impacts on reproductive performance in relation to THI can all be used as selection criteria for heat tolerance. However, for practical purposes, the reduction in yield as THI rises has generated the most interest. This trait is likely to be indicative of animals suffering from heat stress, and also it has a direct economic impact on the farmer, so its value can be quantified economically.

Ravagnolo, Misztal and Hoogenboom (2000) showed how daily milk yield and THI data from weather stations can be combined to estimate genetic variation in reduction in milk yield as THI increases. Two common models to quantify heat tolerance are the "broken stick" model (Bernabucci *et al.*, 2014) and a reaction norm model (Nguyen *et al.*, 2016; Carabaño *et al.*, 2019). The "broken-stick" model first calculates milk production traits under thermo-neutral conditions, and then estimates the slope of the reduction in milk production traits after exposure to heat stress (Bernabucci *et al.*, 2014). The reaction norm model is more flexible and uses polynomials to model a non-linear relationship between THI and the reduction in milk yield, resulting in a steeper slope (i.e. variability in the slope) when the THI increases (Nguyen *et al.*, 2016; Carabaño *et al.*, 2019).

Nguyen *et al.* (2016) obtained genomic prediction accuracies of between 0.42 and 0.61 for heat tolerance by using random regression model and a THI threshold of 60 (20 °C and 45 percent relative humidity). After increasing the size of the reference population, the world's first breeding values for dairy cow heat tolerance were released in 2017 (Nguyen *et al.*, 2017). Currently, DataGene (the national genetic evolution unit for dairy cattle in Australia) reports reliabilities for bulls without progeny of approximately 40 percent.

Understanding the physiological effects of these sorts of selection strategies is important to convince researchers and farmers alike that the EBV is doing what it is designed to do. With this in mind, the heat tolerance genomic EBVs were validated experimentally before being released to farmers. Animals with high and low heat tolerance genomic EBVs obtained by Nguyen *et al.* (2016) had significant differences in milk yield losses ($P < 0.023$ at day 4), rectal ($P < 0.01$ days 2–4) and vaginal ($P < 0.01$ on all 4 days) temperatures under a 4 day climate controlled challenge designed to mimic a mild heatwave in south eastern Australia (Garner *et al.*, 2016). Thus, it appears heat tolerant cows are able to maintain a cooler core body temperature.

Currently, heat tolerance is not included in Australia's national selection index (BPI). Australia is a large country with a large variation in climates. Therefore, the

economic value of selection for improved heat tolerance varies between regions, making it challenging to include heat tolerance in the BPI. Nguyen *et al.* (2017) tested the potential of a region-specific BPI, that used levels of heat load for various regions of Australia as weights for heat tolerance. The region-specific BPI that included weights appropriate for a high THI region was strongly correlated with the BPI (r = 0.95), and consequently only limited re-ranking of sires occurred between the two indices. Therefore, Nguyen *et al.* (2017) concluded that region specific BPIs were not necessary. However, it is important to give farmers the opportunity to select for heat tolerance. Farmers can use the Good Bulls App (a free tool available for Apple and Android devices on app stores, e.g. https://apps.apple.com/au/app/good-bulls/id1066608067) to filter on selection objectives and select simultaneously on the BPI and traits of interest to the farmer that are not in the BPI, such as heat tolerance.

Novel phenotypes for heat tolerance

Sensor technology can provide alternative measures of heat tolerance. Possible sensors include tympanic temperature measurements that are worn on the outside of the cow, and panting scores (Gaughan *et al.*, 2008) that can be measured using accelerometers. The value of the latter trait is questionable, however. While Bar *et al.* (2019) reported that accelerometer measures showed a good concordance with vaginal temperatures, results by Garner *et al.* (2016) found that some cows had very high body core temperatures but only slightly elevated respiratory rates. This was especially true for heat susceptible cows, because they have less efficient evaporative cooling systems.

Internal (adapted intra-vaginal or rectal) devices may be able to record body temperature continuously. The rectal temperatures of cows exposed to heat stress has a heritability of 0.17 (Dikmen *et al.*, 2012). However, such devices can only be used for up to around 14 days and are relatively expensive. Consequently, they are currently not suitable for the large-scale routine measurements required for accurate genomic prediction. There are more expensive alternatives that allow longer recording periods, such as surgically implantable temperature measurement devices, both loggers that need to be extracted from the animal, and loggers that use telemetry to transmit data. Another promising method are ruminal telemetric boli. The temperature in the rumen is approximately 0.5–0.6 °C greater than a cow's rectal temperature (AlZahal *et al.*, 2011; Timsit *et al.*, 2011). While ruminal boli are less invasive than some of the previously mentioned methods, they are only suitable for single use, except in fistulated cows. Furthermore, their measurements may be influenced by water intake (Bewley *et al.*, 2008; Cantor, Costa and Bewley, 2018).

The increased uptake of wearable devices is promising for the collection and development of large datasets that can be used to estimate breeding values for heat tolerance. However, there are a number of challenges that need to be overcome. As described by Gengler (2019), these challenges include: 1) data quality and ensuring the sensor is validated on measured behaviours; 2) data accessibility and availability off-farm; and 3) defining phenotypes from sensors.

Metabolites could also serve as selection criteria that may be related to heat stress. When cows are exposed to heat stress, the breakdown of whole-body protein and urea synthesis increases, indicating the mobilisation of amino acids to meet the cow's requirements (Ríus, 2019). However, it is challenging to obtain sufficient metabolite phenotypes for genetic evaluation. An alternative is to use MIR spectroscopy of a milk sample, which has been shown to detect metabolites associated with heat tolerance (König and May, 2019). Hammami *et al.* (2015) reported associations between several MIR predicted milk fatty acids and heat stress. A particularly strong relationship was observed for C18:1 cis-9, a milk fatty acid related to body tissue mobilization. This result suggested that cows mobilize proportionately more body reserves when they have depressed appetites and yields due to heat stress.

4. Conclusions

Dairy production both contributes to and is affected by climate change. We have described practical genetic solutions for breeding for reduced emissions and increased heat tolerance in dairy cattle. Uptake of EBVs for these important traits could be part of a multi-pronged mitigation strategy to reduce the effects of livestock on global warming in addition to improving resilience of dairy cattle to inevitable climate change.

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Description of gastrointestinal nematode infections by faecal egg count in a carpet wool sheep breed

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Abstract

The ongoing spread of anthelmintic resistance is causing an increasing failure of existing chemical control methods against gastro-intestinal nematodes (GIN). Breeding for genetic resistance of the host is among complementary investigated solutions to anthelmintic use. The most utilized trait used in traditional selection of sheep in favour of GIN resistance is faecal egg count (FEC), the number of eggs per gram of faeces. The aims of this paper are to describe the distribution of nematodes in different flocks of a carpet wool sheep breed to investigate the relationship between different traits related to gastrointestinal nematode resistance. We investigated 211 weaned lambs (age 4–6 months) from nine flocks of the Qizil sheep breed (~20 per flock) from the East Azerbaijan province, Tabriz, the Islamic Republic of Iran. Body weight, FEC, FAMACHA test and packed cell volume were measured. Nematode eggs were classified according to the species or group of species of origin: (1) Strongyles; (2) *Nematodirus* spp.; (3) *Trichuris* sp.; and (4) *Marshallagia marshalli*. We evaluated the correlation among traits and the distribution of the different nematode species among flocks.

Keywords

resistance, sheep, nematodes, parasites, regression, carpet wool breeds

1. Introduction

The number of sheep in the world is 1.2 billion, of which nearly 4 percent are raised in the Islamic Republic of Iran (FAO, 2017). The Qizil (or Ghezel) is a fat-tailed sheep breed mainly reared in the northwest regions of the Islamic Republic of Iran and in eastern and north-eastern regions of the Republic of Türkiye, with an estimated population size of 17 million heads, four of which are reared in the Islamic Republic of Iran (DaskİIran and Ayhan, 2014). Qizil sheep are mostly reared on open pasture where gastrointestinal nematodes (GIN) are common. Anthelmintic treatments against GIN exist and are commonly applied to combat the infestation, but drug resistance is increasingly reported. McMahon *et al.* (2017) observed a reduced efficacy of benzimidazole, avermectins and moxidectin treatment in northern region of the Republic of Iceland. Drug resistance to levamisole and albendazole in sheep flocks of the Islamic Republic of Iran have been reported in 66 and 27 percent of

cases, respectively. Unfortunately, alternative methods of chemical control against GIN are not currently available. Moreover, environmental problems associated with chemical control of parasites are notably important for consumers (Charlier *et al.*, 2018).

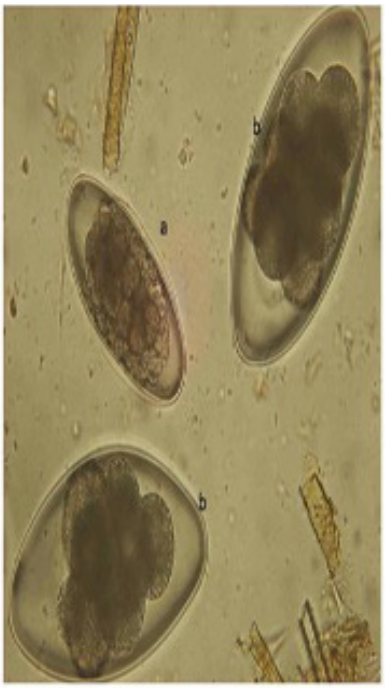
Breeding for host genetic resistance is a potential GIN control strategy (Preston *et al.*, 2014). The number of eggs per gram of faeces (EPG) is frequently used as selection criteria for resistance or resilience to GIN. EPG has low heritability, but selective breeding has been shown to be effective in improving the trait and reducing EPG values. It is to be noted that the use of EPG in sheep breeding programmes has some limitations. One of the limitations is the need to have some infected animals to record variability in the trait. In addition, variations in natural GIN infection and environmental load depend on climatic conditions that may vary across pastures, seasons and years. Further, measurement of EPG is labour intensive. Therefore, the availability of genetic markers associated to GIN resistance/tolerance is highly desirable. Markers would permit assisted selection for genetic resistance by using a drug-free and less labour-intensive strategy (Atlija *et al.*, 2016) to be applied as a part of an integrated parasite management strategy for the control of GIN.

This investigation is part of the Coordinated Research Project sponsored by IAEA on "Genetic variation on the control of resistance to infectious diseases in small ruminants for improving animal productivity genetic resistance to nematodes in small ruminants." Objectives of this paper are to describe the distribution of GIN groups in different flocks of a carpet wool breed. and to investigate the relationships among traits associated with GIN resistance.

2. Material and methods

Animals and phenotypes

This study was conducted during the month of May, when rainfall and humidity were seasonally high, and animals were thus at the highest risk of becoming contaminated with GIN (based on past anecdotal observations). We selected 211 individuals among weaned lambs (age 4–6 months) from nine flocks (approximately 20 individuals per flock) of the Qizil sheep breed in East Azerbaijan province, Tabriz, the Islamic Republic of Iran. Body weight, faecal egg count, FAMACHA (FAffa MAlan CHArt) test and packed cell volume (PCV) were measured twice at one-week intervals. Nematode eggs were classified into four groups according to the species or group of species of origin: (1) Strongyles (EPGO); (2) *Nematodirus* spp. (EPGN); (3) *Trichuris* sp. (EPGT); and (4) *Marshallagia marshalli* (EPGM). Photo S1 shows Strongyles (*Trichostrongylus* spp.) and *Nematodirus* spp. The sum of the mass of eggs from the four nematode groups in each lamb's faeces are reported as EPG. FAMACHA is an ordinal trait with five levels and is based on the colour of the inner eyelids of sheep. Sheep with a darker red colour are considered healthy and are assigned low scores, whereas sheep with lighter inner eyelids colour are assumed to be anaemic due to nematode infection and are assigned higher scores as the degree of redness decreases.



Source: Authors' own elaboration.

Photo S1: a) *Trichostrongylus* spp.; b) *Nematodirus* spp

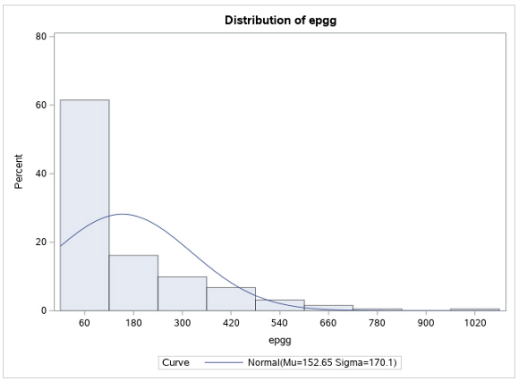
Statistical analyses

Correlation coefficients between traits were calculated by PROC CORR of SAS (SAS STUDIO). The distribution of traits was verified by PROC UNIVARIATE of SAS. Data of EPGO, EPGN, EPGT, EPGM and EPG were submitted to Box–Cox transformation by PROC TRANSREG to approximate a normal distribution (Figure 1). For these traits, analysis of variance was performed on the transformed data, while means are presented as non-transformed. Numbers equal to zero were replaced by 1 to enable transformation by the Box-Cox method. Distributions of PCV and FAMACHA are presented in Figure 2.

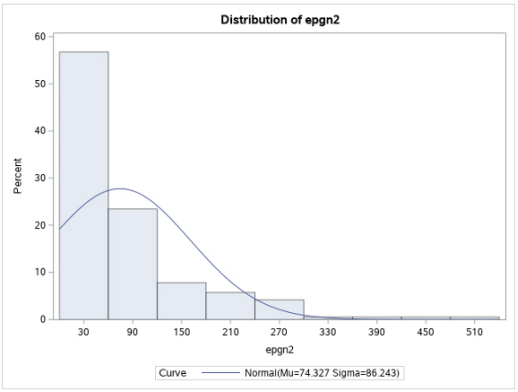
Initial analysis of variance was performed to investigate the effects of sex, flock, and live body weight (as linear covariate) on PCV, EPGO, EPGN, EPGT and EPG. Only the effect of flock resulted significant, so the other effects were removed from the model. FAMACHA scores were analysed by PROC FREQ of SAS with chi-square test.

We investigated the relationship between EPGO as dependent variable and FAMACHA as an independent variable using Proc REG of SAS. We estimated regression coefficients within each flock.

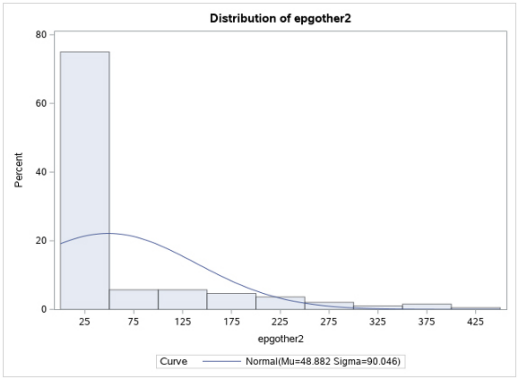
EPG



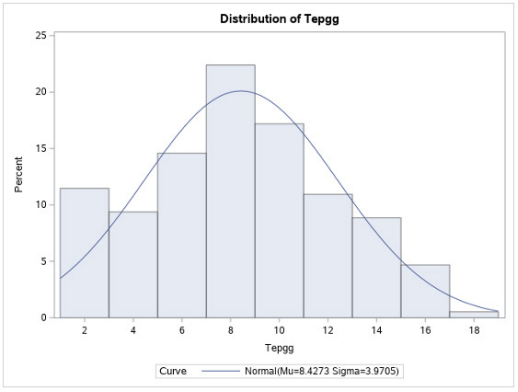
EPGN



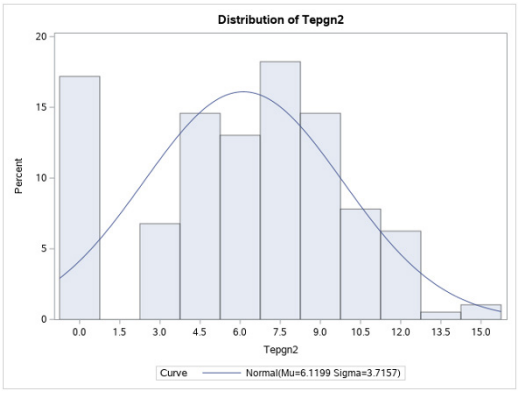
EPGO



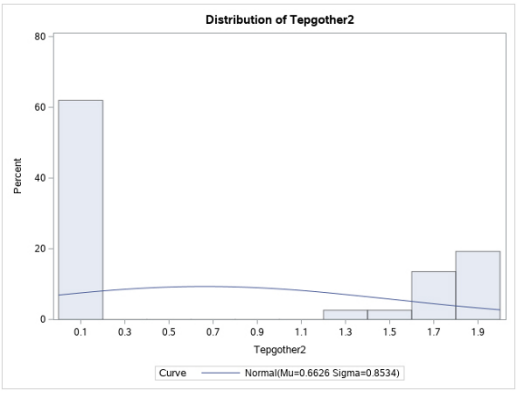
TEPG



TEPGN

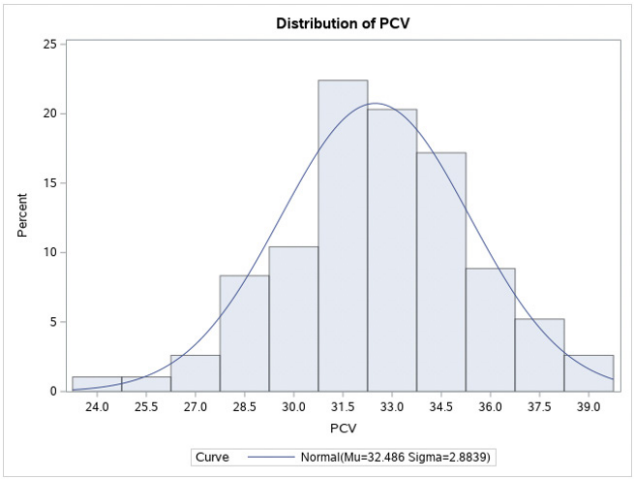


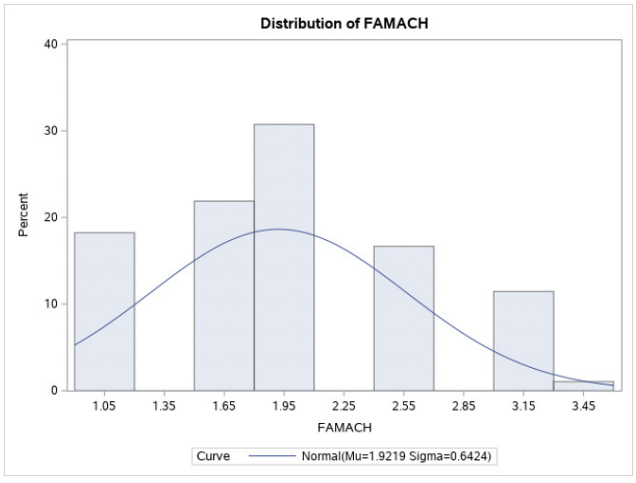
TEPGO



Source: Authors' own elaboration.

Figure 1: Non-transformed (EPG = overall eggs per gram of faeces; EPGN = EPG of *Nematodirus* spp.; EPGO = EPG of Strongyles spp.) and Box-Cox transformed (TEPG, TEPGN and TEPGO) distributions





Source: Authors' own elaboration.

Figure 2: Distribution of the PCV (left panel) and FAMACHA (right panel) traits

3. Results and discussions

Descriptive statistics of phenotypic traits are presented in Table1. The Pearson correlation coefficient between EPG and PVC was 0.30 ($P < 0.0001$). Pearson and Spearman correlations between PCV and FAMACHA were -0.49 and -0.47, respectively ($P < 0.0001$).

Distribution of FAMACHA score among flocks is shown in Table 2. The chi-square test showed an association between the FAMACHA score and flock ($P < 0.03$).

The effect of flock resulted significant in all ANOVA tests run on the different traits. A comparison of means is presented in Figure 3. Previous studies have reported parasitic variability among flocks (Falzon *et al.*, 2013) and geographic areas (Papadopoulos *et al.*, 2003). Importantly, previous studies have observed that climatic conditions can have a great effect on the prevalence and severity of GI nematode parasites (Nabavi *et al.*, 2011).

Nabavi *et al.* (2011) attributed the greatest number of abomasal GIN infections among species to *Teladorsagia circumcincta*, as we observed in Flocks 3 and 5 (Figure 4). There is high variability among flocks for overall level of infection and for the relative importance of the different GIN species (Figure 4). For example, Flocks 5 and 8 showed very different levels of EPG. This likely reflects differences in parasite load in the environment between these flocks. This might be due to differences in management factors (e.g. routine deworming, stall fed vs grazing, etc.) or in climatic conditions (e.g. rainy/dry weather, etc.) across flocks. For example, a positive correlation has been reported to exist between EPG and the mean humidity (Nematollahi *et al.*, 2020). Dominik and colleagues (Dominik, 2005) reviewed genetic variation within flocks of the same breeds for resistance to GIN, and Becker *et al.* (2020) observed phenotypic variation in natural helminth infection. In our study, flock genetic makeup is unlikely to contribute substantially to between flock phenotypic variation in EPG, considering the low heritability of the trait. Furthermore, this hypothesis would imply a high proportion of the genetic variance

Table 1: Descriptive statistics of the studied GIN related traits

Variable	*n*	Min	Max	Mean	SD
EPG#	192	4	1 071.50	152.65	170.1
EPGN	192	1	526.00	74.33	86.24
EPGM	192	1	219.75	26.94	42.91
EPGT	192	1	44.75	2.50	6.03
EPGO	192	1	447.25	48.88	90.05
PCV	192	23.50	39.25	32.49	2.88
FAMACHA	192	1	3.5	1.92	0.64

Source: Authors' own elaboration.

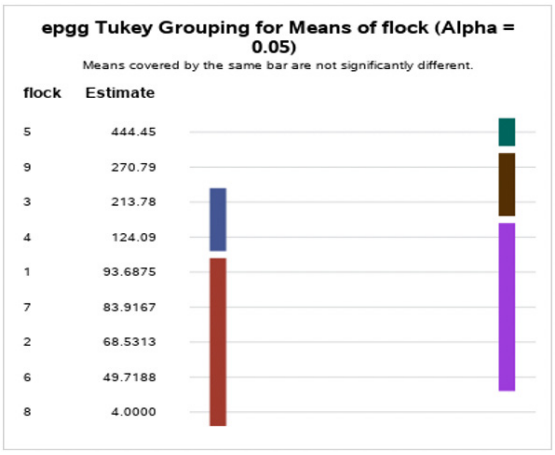
Note: **PCV**: Packed Cell Volume, **EPGO**: EPGal egg count for Strongyles including *Haemonchus contortus, Teladorsagia circumcincta, Ostertagia occidentalis, Trichostrongylus axei, colubriformis, vitrinus, and rugatus*, **EPGN**: *Nematodirus* spp., **EPGT**: *Trichuris* sp., **EPGM**: *Marshallagia marshalli*. The sum of eggs in the four classes in each lamb's faeces were reported as **EPG**.

Table 2: Frequency of different FAMACHA scores in each flock and contribution in percent of each flock to the frequency across flocks

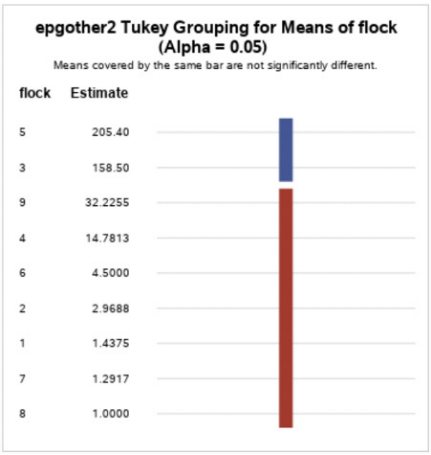
FAMACHA	Flock									
	1	2	3	4	5	6	7	8	9	Total
1	3 1.56	2 1.04	7 3.65	7 3.65	5 2.60	3 1.56	5 2.60	3 1.56	0 0.00	**35** **18.23**
1.5	7 3.65	9 4.69	5 2.60	7 3.65	2 1.04	1 0.52	3 1.56	3 1.56	5 2.60	**42** **21.88**
2	8 4.17	7 3.65	4 2.08	2 1.04	8 4.17	4 2.08	11 5.73	7 3.65	8 4.17	**59** **30.73**
2.5	1 0.52	2 1.04	2 1.04	4 2.08	4 2.08	7 3.65	6 3.13	3 1.56	3 1.56	**32** **16.67**
3	1 0.52	0 0.00	2 1.04	0 0.00	5 2.60	5 2.60	5 2.60	3 1.56	1 0.52	**22** **11.46**
3.5	0 0.00	0 0.00	0 0.00	0 0.00	1 0.52	0 0.00	0 0.00	1 0.52	0 0.00	**2** **1.04**
Total	**20** **10.42**	**20** **10.42**	**20** **10.42**	**20** **10.42**	**25** **13.02**	**20** **10.42**	**30** **15.63**	**20** **10.42**	**17** **8.85**	**192** **100.00**

Source: Authors' own elaboration.

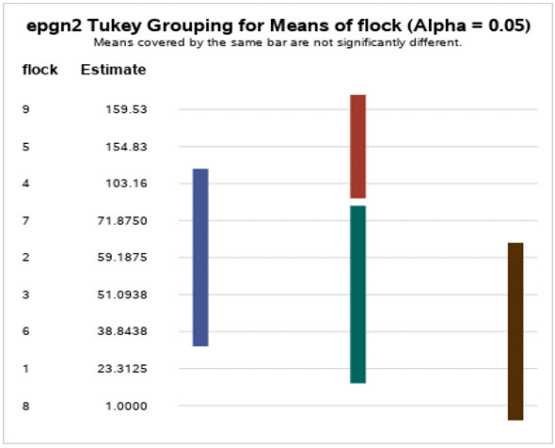
EPG



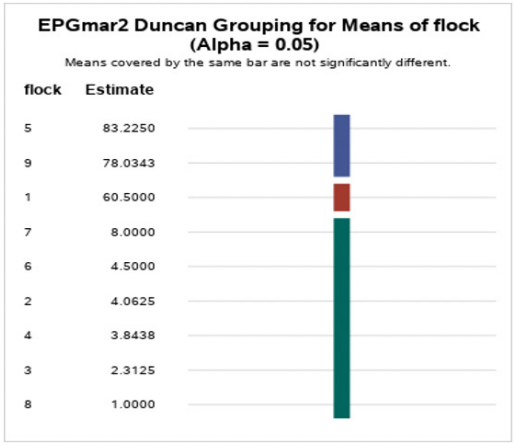
EPGO



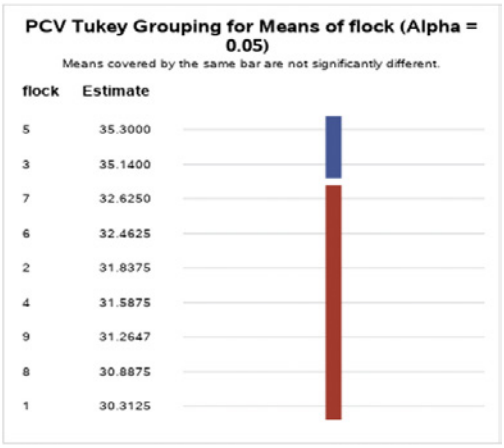
EPGN



EPGM



PCV



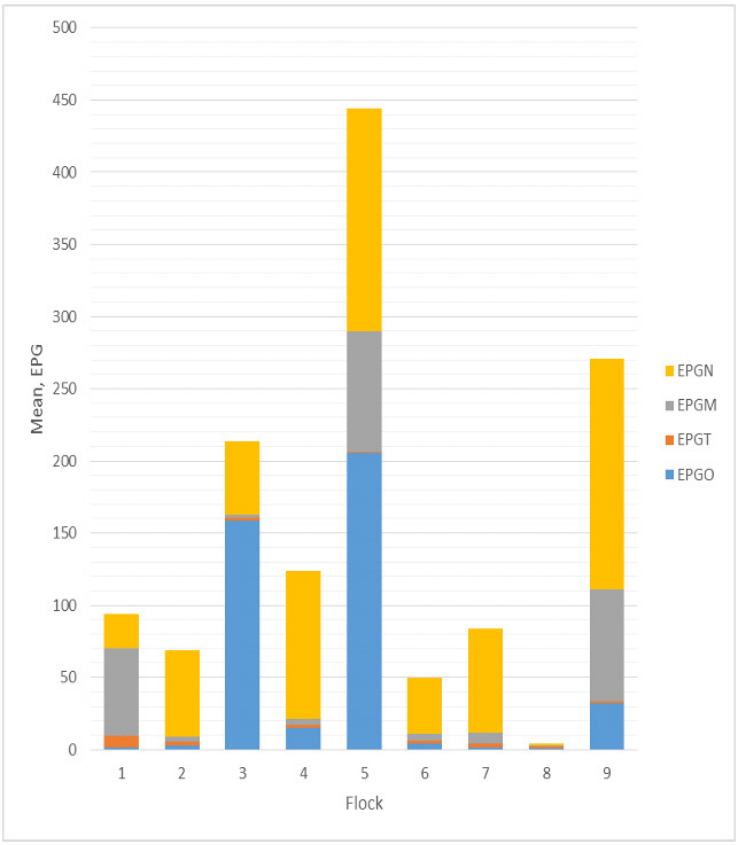
Notes: a Means covered by the same bar are not significantly different (Tukey grouping, alpha = 0.05). For example, for EPG, the mean for Flock 5 is significantly greater than for all other flocks. Flock 9 differs from all flocks except for Flock 3, although Flock 3 does not differ significantly from Flock 4. Meanwhile, the mean of Flock 4 does not differ from the means of Flocks 1,7, 2 and 6. Finally, the means of Flocks 1,7,2,6 and 8 do not differ significantly.

b **EPGO**: EPGal egg count for Strongyles including *Haemonchus contortus, Teladorsagia circumcincta, Ostertagia occidentalis, Trichostrongylus axei, colubriformis, vitrinus, and rugatus*, **EPGN**: *Nematodirus* spp., **EPGT**: *Trichuris* sp., **EPGM**: *Marshallagia marshalli*.

Source: Authors' own elaboration.

Figure 3: Comparison of means among flocks in the studied traits

to occur between flocks, an event occurring very rarely in populations for which no flocks have historically practiced formal directional selection for GIN resistance. Conversely, we cannot exclude the presence of a genetic component of individual response to parasite challenge, observing the high phenotypic variation of animals within flocks, particularly in those having high mean parasitic load. Interestingly, the frequency of different GIN species varied across flocks, independently on total parasite load. For instance, EPGN was present in all flocks, and was the prevalent species in five flocks (2, 4, 6, 7 and 9), but not in Flocks 3 and 5, in which the prevalent species is EPGO. Also, EPGM was almost absent in Flock 3, which had an overall high parasite load, while it was in a high proportion for Flocks 5 and 9, which had the two highest overall parasitic loads, but also in Flock 1 that had a medium load. The reasons for these differences are unclear and may depend on local climatic conditions as indicated above.



Note: **EPGO**: EPGal egg count for Strongyles including *Haemonchus contortus, Teladorsagia circumcincta, Ostertagia occidentalis, Trichostrongylus axei, colubriformis, vitrinus, and rugatus*, **EPGN**: *Nematodirus* spp., **EPGT**: *Trichuris* sp., **EPGM**: *Marshallagia marshalli*.
Source: Authors' own elaboration.

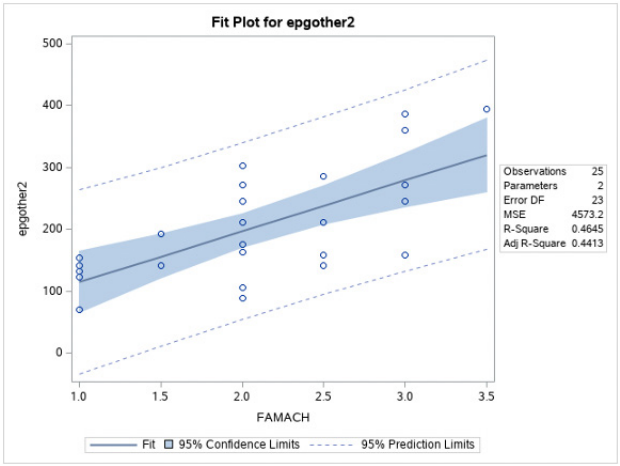
Figure 4: GI distributions among flocks

The regression analysis between traits within flocks resulted in coefficients (slopes) having different values for the same comparison, likely due to the very variable parasite loads among flocks and the limited numbers of animals per flock. In only a few flocks were the regression coefficients significant with an acceptable coefficient of determination (R-square > 0.4). Plots of EPGO and PCV (as dependent variables) on FAMACHA (as independent variable) are shown in Figure 5. The regression equations were:

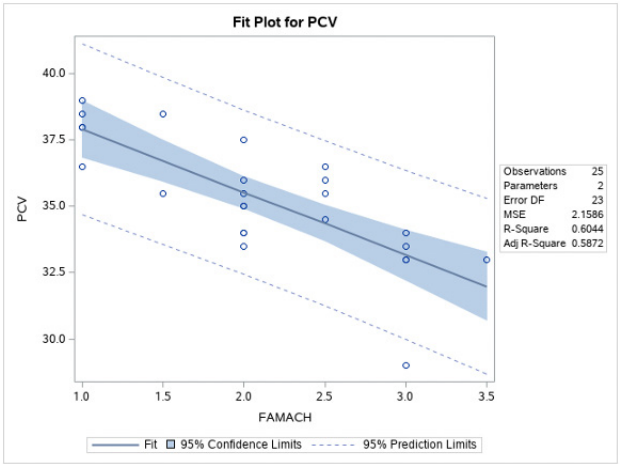
EPGO = 32.74 + 82.22 FAMACHA

PCV = 40.28 - 2.37 FAMACHA

EPGO



PCV



Source: Authors' own elaboration.

Figure 5: Fitted linear model (solid line) of EPGO (left) and PCV (right) as predicted by FAMACHA within flock No 5. Circles are the original observations, and the blue shaded area represent the Confidence Intervals (95 percent) of the model

4. Conclusions

In general, it can be said that there is diversity within and between flocks in terms of GIN species infections. Given that effects of body weight and sex were not significant, this diversity may primarily be due to management and climatic conditions. Genetic effects cannot be ruled out, however, as different parasite loads have been observed in lambs from a same flock. The diversity between individuals deserves further study, to understand the genetic basis of resistance or susceptibility to GIN in the breed investigated.

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Performance of Mecheri lambs introgressed with *FecB* gene in tropical climatic conditions of Tamil Nadu, India

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Abstract

Study on growth performance of crossbred Mecheri lambs introgressed with the fecundity gene (*FecB*) was carried out at the Mecheri Sheep Research Station, Pottaneri, Tamil Nadu, India. The overall least-squares means (±SE) for body weight of crossbred Mecheri lambs at birth, 3, 6, 9 and 12 months of age were 2.296 ± 0.04, 9.60 ± 0.23, 13.72 ± 0.32, 15.62 ± 0.32 and 18.95 ± 0.37 kg, respectively. The body weights in the F_1 generation were higher than those observed in F_2 generations in all age groups and the differences were significant ($p < 0.05$) at birth and 3 months of age. The sex of the lamb had a significant ($p < 0.05$) effect on body weight at six, nine and 12 months of age. The difference between male and female lambs increased from 0.043 kg at birth to 4.41 kg at 12 months of age. The least-squares means (±SE) for daily body weight gain of ram lambs at 0 to 3 months, 3 to 6 months and 6 to 12 months were 81.52 ± 3.79, 54.67 ± 4.66 and 40.41 ± 3.41 g, respectively, and the corresponding values for females were 77.59 ± 3.70, 38.65 ± 3.70 and 25.46 ± 2.13 g, respectively. The percentages of mortality in young and adult crossbred Mecheri sheep were 51.28 (20 lambs out of total 39 animals) and 48.72 percent (19 adult sheep out of total 39 animals), respectively. The percentages of crossbred Mecheri sheep that died due to acute bloat, enteritis, plant poisoning, pneumonia, debility, poor birth weight, hooked tooth, toxaemia, hepatitis, impaction due to polythene bag obstruction, intestinal rupture and enterotoxaemia were 5.1, 12.8, 5.1, 2.6, 2.6, 28.2, 17.9, 5.1, 2.6, 5.1, 2.6 and 10.3 percent, respectively. The tupping, lambing (based on ewes tupped) and twinning percentages observed in Mecheri crossbred ewes carrying the *FecB* gene (both homozygous and heterozygous) were 68.49, 78.04 and 32.25 percent, respectively. The proportions of animals sold for breeding and slaughter were 28.40 and 71.60 percent, respectively.

Keywords

Mecheri, sheep, crossbred, fecundity, growth performance

1. Introduction

Sheep in India primarily provide meat, fibre and manure; and their skins are a valuable by-product. In general, reproductive ability has important impacts on profitability of farm animals and is measured by fertility, prolificacy and fecundity. Genomics has offered the opportunity to identify major genes associated with ovulation rate in sheep including the *FecB* gene (Piper, Bindon and Davis, 1985). On an average, one copy of the *FecB* allele increases ovulation rate by 1.2 ova shed per ewe ovulating, and lambing rate by 0.6 lambs born ewe lambing (Piper, Bindon and Davis, 1985). In addition to the *FecB* gene, a number of major prolificacy genes viz., BMPR1B (bone morphogenetic protein receptor type 1B), BMP15 (bone morphogenetic protein 15), GDF9 (growth differentiation factor 9) and B4GALNT2 (Beta-1,4-N-Acetyl-Galactosaminyltransferase 2) located on ovine chromosomes 6, X, 5 and 11, respectively, have been identified in sheep (Galloway *et al.,* 2000; Souza *et al.,* 2001; Hanrahan *et al.,* 2004; Drouilhet *et al.,* 2013).

India is the seventh largest country in the world in terms of land mass. Due to its diverse agro-ecological regions and topographic conditions, India has a rich repository of both flora and fauna. Sheep biodiversity in India is characterized by a high degree of endemism, and variations in the agro-climatic conditions have led to the development of various breeds/strains that are well adapted to specific set of local environmental conditions (Thiruvenkadan

et al., 2017). Sheep play an important role in the Indian agrarian economy and are maintained mainly on natural vegetation on community grazing lands, fallow lands and uncultivated lands, as well as the stubble of cultivated crops and high-quality feeds. The individual breeds are very well adapted to the harsh climatic conditions prevailing in their particular breeding tract as well as to the prevailing tropical diseases.

Mecheri sheep are one among the ten recognized breeds of sheep in Tamil Nadu and are reared mainly for meat; the primary by-product is skin. Figure 1 shows a flock of Mecheri sheep in a typical grazing environment. The Mecheri sheep breed is known for its excellent meat and skin quality and its adaptation to harsh climatic conditions (Karunanithi *et al.,* 2005). A nucleus flock of Mecheri sheep has been established at the Mecheri Sheep Research Station and selective breeding is practised to improve the performance of the flock mainly for body weight. Elite germplasm is distributed locally for genetic improvement in farmers flocks. Mutton is a widely consumed meat in the Tamil Nadu and across India in general, and the demand for mutton is progressively increasing in view of substantial increases in per capita income. Therefore, to meet the increasing demand and also to boost the livelihoods of small and/or marginal farmers, there is an urgent need to increase productivity of the sheep for meat production. To increase the productivity through enhancing the fecundity in Mecheri sheep, a genetic improvement programme based on introgression of the *FecB* gene has been implemented. This paper assesses the status and performance of this introgressed population under tropical climatic conditions of southern part of India.

Source: Authors' own elaboration.

Figure 1: A flock of Mecheri sheep in its typical production environment (photo provided by A.K. Thiruvenkadan)

2. Materials and methods

Data on the performance of 236 Mecheri crossbred lambs (131 males and 105 females), maintained at Mecheri Sheep Research Station (MSRS), Pottaneri, Salem, Tamil Nadu, India, were collected over a period of four years (2013–2016). The MSRS is located in the dry land farming condition of Tamil Nadu, where the climate is generally hot, semi-arid and tropical in nature. The maximum temperature recorded is 37.4 ℃ (in the month of May) and the minimum temperature recorded is 18.5 ℃ (in the month of January). The average annual rainfall is about 975 mm, and the monsoon season starts in June and lasts until the end of November.

The Mecheri sheep in this study were reared under a semi-intensive system of management, and all animals grazed during the day (7 to 8 h) on natural pasture with supplementation depending upon the status and age category of the animals, and were penned at night. Lambs were normally weaned at three months of age. Adult animals were vaccinated against enterotoxaemia, Foot-and-Mouth Disease, Bluetongue, *peste des petits Ruminants* (PPR) and sheep pox and were also treated against internal and external parasites. Lambs were vaccinated against enterotoxaemia at two months of age, with a booster one month later and drenched with deworming medicines at the time of weaning. Mineral blocks were always available for all the animals.

In purebred Mecheri sheep population, the genetic introgression of *FecB* gene was made in the year 2013 and the production of F_1 animals was achieved through crossing purebred Mecheri sheep (n = 87) with Deccani crossbred rams (using six rams carrying homozygous (BB) genotype). The F_1 crossbred Mecheri ewes were again mated with the foundation stock rams to produce F_2 crossbreds; the mating of F_1 was made in such a way to avoid inbreeding in the flock. The genotype at the *FecB* locus was identified by molecular techniques. The body weights of these crossbred lambs at different ages (i.e. at birth), weaning (i.e. at 3 months), 6, 9 and 12 months were collected from the birth and growth registers maintained in the farm. The data were classified according to period, season, type of birth, generation, parity of dam and sex, and analysed using least-squares analysis of variance (Harvey, 1990). In addition, the data on mortality, causes of death and disposal particulars were collected and analysed using standard statistical procedures (Snedecor and Cochran, 1989).

3. Results

Body weight at different ages and effect of non-genetic factors

The least-squares means (±SE) for body weight of Mecheri crossbred lambs are presented in Table 1. The Overall least-squares means for body weight at birth, 3, 6, 9 and 12 months of age were 2.296 ± 0.04, 9.60 ± 0.23, 13.72 ± 0.32, 15.62 ± 0.32 and 18.95 ± 0.37 kg, respectively. The number of individuals observed at the age of one year was 73.31 percent lower than the number of lambs observed at birth. The reasons for this reduction were mortality and culling of unproductive lambs as well as sale or transfer of animals. The study on non-genetic factors on body weight at different ages revealed that the body weight observed in the F_1 generation were higher than those observed in F_2 generations in all the years and the differences were significant at birth and 3 months of age alone. The higher body weight observed in F_1 generations might be due to hybrid vigour. The sex of the lamb had a significant (p < 0.05) effect on 6, 9 and 12 month body weight. Comparison

Table 1: Least-squares means (±se) for body weight of mecheri crossbred lambs

Effects	Birth	Three months	Six months	Nine months	12 months
Overall	2.296 ± 0.04 (236)	9.60 ± 0.23 (161)	13.72 ± 0.32 (109)	15.62 ± 0.32 (95)	18.95 ± 0.37 (63)
Generation	*	*			
F1	2.420 ± 0.05^{b} (199)	10.022 ± 0.25^{b} (141)	13.82 ± 0.33 (104)	15.75 ± 0.34 (90)	19.07 ± 0.37 (62)
F2	1.883 ± 0.08^{a} (37)	7.97± 0.54^{a} (20)	12.57 ± 1.27 (5)	14.42 ± 1.19 (5)	16.30 ± 0.00 (1)
Sex			*	*	*
Male	2.317 ± 0.05 (131)	9.80 ± 0.32 (78)	14.60 ± 0.52^{b} (38)	16.12 ± 0.59^{b} (27)	22.40 ± 0.78^{b} (14)
Female	2.274 ± 0.06 (105)	9.40 ± 0.32 (83)	13.11 ± 0.41^{a} (71)	15.41 ± 0.39^{a} (68)	17.99 ± 0.42^{a} (49)
Type of birth	**				
Single	2.471 ± 0.05^{a} (203)	9.75 ± 0.26 (135)	13.98 ± 0.36 (95)	15.93 ± 0.35 (83)	19.36 ± 0.40 (55)
Twin	1.926 ± 0.07^{b} (33)	9.16 ± 0.47 (26)	12.97 ± 0.72 (16)	14.55 ± 0.80 (12)	17.48 ± 0.91 (8)
Year					
2013	2.559 ± 0.11 (33)	9.31 ± 0.50 (31)	14.87 ± 0.59 (31)	16.40 ± 0.60 (30)	19.90 ± 0.54 (27)
2014	2.433 ± 0.08 (83)	10.03 ± 0.35 (81)	12.88 ± 0.47 (57)	15.21 ± 0.47 (46)	18.25 ± 0.53 (33)
2015	2.184 ± 0.07 (49)	9.91± 0.48 (31)	13.44 ± 0.71 (20)	15.31 ± 0.68 (19)	17.60 ± 1.27 (3)
2016	2.136 ± 0.07 (71)	8.85 ± 0.56 (18)	17.30 ± 0.00 (1)	-	-
Season	**				
Off season	2.049 ± 0.07^{a} (64)	8.93 ± 0.44 (38)	13.34 ± 0.81 (16)	14.87 ± 0.86 (11)	19.73 ± 0.90 (16)
Main season	2.438 ± 0.05^{b} (172)	9.90 ± 0.26 (123)	13.79 ± 0.35 (93)	15.73 ± 0.35 (84)	18.92 ± 0.38 (57)
Parity	**				
First	2.018 ± 0.06^{a} (104)	8.94 ± 0.36 (70)	13.03 ± 0.54 (48)	15.41 ± 0.55 (42)	17.62 ± 0.62 (28)
Second	2.224 ± 0.08^{ab} (70)	8.88± 0.51 (47)	13.57 ± 0.62 (31)	15.14 ± 0.58 (28)	17.36 ± 0.85 (19)
Third	2.434 ± 0.09^{ab} (38)	9.91 ± 0.53 (26)	14.61 ± 0.78 (18)	15.43 ± 0.76 (16)	19.53 ± 1.03 (10)
Fourth	2.808 ± 0.18^{b} (8)	10.67 ± 0.88 (6)	14.45 ± 1.53 (4)	16.95 ± 1.43 (3)	21.02 ± 1.10 (2)
Fifth	2.625 ± 0.17^{ab} (8)	10.07 ± 0.78 (6)	14.11 ± 1.06 (4)	14.63 ± 1.09 (3)	19.91 ± 1.03 (2)
Sixth	2.588 ± 0.17^{b} (8)	11.42 ± 0.87 (6)	13.40 ± 1.14 (4)	18.05 ± 1.35 (3)	20.25 ± 1.27 (2)

Source: Authors' own elaboration.

Notes: a Figures in parentheses are number of observation.
b * (P < 0.05); ** (P < 0.01).

of the least-squares means for lamb weights at different ages showed that the difference between male and female lambs increased from 0.043 kg at birth to 4.41 kg at 12 months of age. In general, the year of lambing had no significant effect on body weight of the lambs at most of the developmental stages. The lambs born as singles had higher body weights than those born as twins and were significantly ($p < 0.01$) heavier at birth. The parity of the dam had a highly significant ($p < 0.01$) effect on the body weight at birth only. In general, the body weight increased with the advancement of dam parity and the lambs born from the 3rd and 4th parities maintained a higher body weight at all ages.

Body weight gain at different ages

The least-squares means (±SE) for average daily body weight gain (ADG) of ram lambs at 0 to 3 months (ADG1), 3 to 6 months (ADG2) and 6 to 12 months (ADG3) were 81.52 ± 3.79, 54.67 ± 4.66 and 40.41 ± 3.41 g, respectively, and the corresponding values for females were 77.59 ± 3.70, 38.65 ± 3.70 and 25.46 ± 2.13 g, respectively (Table 2). The ADG values observed in the F_1 generation were higher than those observed in F_2 generations in all the years, but were significant for ADG1 only. The sex of the lamb had a significant ($p < 0.05$) effect on ADG3. In general, the year of lambing had a significant effect ($p < 0.05$) on ADG2 and ADG3. The type of birth and season of lambing had no significant effect on ADG at different stages.

Reproduction performances

The reproduction performance of purebred and crossbred Mecheri ewes over the periods is presented in Table 3. The mean tupping, lambing and twinning percentages observed in Mecheri purebred ewes crossed with rams carrying *FecB* gene (homozygous) were 94.26, 84.65 and 7.14 percent, respectively, and the corresponding values for their crossbred Mecheri progeny carrying the *FecB* gene (both homozygous and heterozygous) were 68.49, 78.04 and 32.25 percent, respectively. The average litter size at lambing in Mecheri purebred and their crossbred progenies were 1.07 and 1.32, and the increased average litter size in crossbred progeny might be due to increased fecundity of the crossbred ewes due to the presence of *FecB* genes.

Table 2: Least-squares means (±se) for average daily weight gain (adg) mecheri crossbred lambs

Effects	Birth to three months ADG1	Three to six months ADG2	Six to 12 months ADG3
Overall	79.59 ± 2.65 (161)	45.46 ± 2.91 (109)	30.06 ± 1.81 (63)
Generation	*		
F1	82.64[b] ± 2.92 (141)	45.00 ± 3.00 (104)	30.30 ± 1.83 (62)
F2	67.05[a] ± 6.26 (20)	51.24 ± 11.56 (5)	23.89 ± 11.08 (1)
Sex			*
Male	81.52 ± 3.79 (78)	54.67 ± 4.66 (38)	40.41[b] ± 3.41 (14)
Female	77.59 ± 3.70 (83)	38.65 ± 3.70 (71)	25.46[a] ± 2.13 (49)
Type of birth			
Single	79.56 ± 3.03 (135)	48.92 ± 3.20 (95)	30.05 ± 1.97 (55)
Twin	79.67 ± 5.43 (26)	35.07 ± 6.55 (14)	30.09 ± 4.26 (8)
Year		*	*
2013	72.19 ± 5.81 (31)	67.49 ± 5.12 (31)	31.31[a] ± 2.58 (27)
2014	83.34 ± 4.06 (81)	29.30 ± 4.27 (57)	31.78[a] ± 2.67 (33)
2015	85.50 ± 5.57 (31)	43.23 ± 6.47 (20)	18.89[b] ± 6.39 (3)
2016	74.46 ± 6.50 (18)	67.78 ± 22.70 (1)	-
Season			
Off season	74.61 ± 5.10 (38)	44.99 ± 7.32 (16)	33.06 ± 4.52 (6)
Main season	81.77 ± 3.08 (123)	45.54 ± 3.16 (93)	29.94 ± 1.87 (57)
Parity	NS	NS	NS
First	76.91 ± 6.80 (70)	45.44 ± 4.68 (48)	25.50 ± 8.52 (28)
Second	73.96 ± 6.76 (47)	52.11 ± 7.63 (31)	21.06 ± 6.55 (19)
Third	83.07 ± 8.64 (26)	52.22 ± 8.25 (18)	27.33 ± 8.24 (10)
Fourth	87.36 ± 10.44 (6)	42.00 ± 8.50 (4)	36.50 ± 6.54 (2)
Fifth	82.72 ± 9.68 (6)	44.89 ± 8.76 (4)	32.22 ± 4.64 (2)
Sixth	98.13 ± 8.88 (6)	22.00 ± 10.20 (4)	38.06 ± 4.60 (2)

Source: Authors' own elaboration.

Notes: a Figures in parentheses are number of observation.
b * ($P < 0.05$); ** ($P < 0.01$).

Table 3: Reproduction performance of purbred and crossbred mecheri ewes

Parameters	Purebred Mecheri ewes mated with homozygous *FecB* crossbred rams			Crossbred Mecheri ewes (F1) mated with homozygous *FecB* crossbred rams		
	Off season	Main season	Overall	Off season	Main season	Overall
Number of ewes allowed	193	51	**244**	32	41	**73**
Number of ewes mated	186	44	**230**	24	26	**50**
Number of ewes lambed	147	35	**182**	11	20	**31**
Number of ewes sold before lambing	0	0	**0**	0	0	**0**
Number of ewes died before lambing	2	0	**2**	1	0	**1**
Number of abortions	5	4	**9**	2	1	**3**
Number of stillbirths	3	3	**6**	3	2	**5**
Number of ewes mated excluding sold, died, abortions and stillbirths	176	37	**213**	18	23	**41**
Number of twins	11	2	**13**	5	6	**10**
Number of lambs born	158	37	**195**	16	27	**41**
Tupping percent (number of ewes mated/number of ewes allowed)	96.37 (186/193)	86.27 (44/51)	**94.26 (230/244)**	75.0 (24/32)	63.41 (26/41)	**68.49 (50/73)**
Lambing percent (number of ewes lambed/number of ewes mated)	83.52 (147/176)	94.59 (35/37)	**85.45 (182/213)**	61.11 (11/18)	91.30 (21/23)	**78.04 (32/41)**
Twining percent (number of twin births/ total number of births)	7.48 (11/147)	5.71 (2/35)	**7.14 (13/182)**	45.45 (5/11)	28.57 (6/21)	**32.25 (10/31)**
Average litter size	1.07 (158/147)	1.05 (37/35)	**1.07 (195/182)**	1.45 (16/11)	1.29 (27/21)	**1.32 (41/31)**

Source: Authors' own elaboration.

Mortality pattern and disposal particulars

The mortality pattern of crossbred Mecheri sheep is presented in Table 4. The percentages of mortality in young and adult crossbred Mecheri sheep were 51.28 (20 lambs out of total 39 animals) and 48.72 percent (19 adult sheep out of total 39 animals), respectively. The percentages of crossbred Mecheri sheep that died due to acute bloat, enteritis, plant poisoning, pneumonia, debility, poor birth weight, hooked tooth, toxaemia, hepatitis, impaction due to polythene bag obstruction, intestinal rupture and enterotoxaemia 5.1, 12.8, 5.1, 2.6, 2.6, 28.2, 17.9, 5.1, 2.6, 5.1, 2.6 and 10.3 percent, respectively. In adult animals, the maximum mortality was observed in crossbred Mecheri ewes (94 .74 percent, i.e. 18 out of 19 adult animals died). The highest lamb mortality was due to poor birth weight and was 55.0 percent of the total lamb mortality (i.e. 11 out of 20 crossbred Mecheri lambs).

Table 4: Mortality pattern of crossbred mecheri lambs

Cause of mortality	No. of animals died	Percentage over total death
Acute bloat	2	5.1
Enteritis	5	12.8
Plant poisoning	2	5.1
Pneumonia	1	2.6
Debility	1	2.6
Poor birth weight	11	28.2
Hooked tooth	7	17.9
Toxaemia	2	5.1
Hepatitis	1	2.6
Polythene bag obstruction	2	5.1
Intestinal rupture	1	2.6
Enterotoxaemia	4	10.3
Total	**39**	**100.00**

Source: Authors' own elaboration.

The sales pattern of crossbred Mecheri lambs and adults is presented in Table 5. The proportions of animals sold for breeding and slaughter were 28.40 and 71.60 percent, respectively. The percentages of young and adult animals that died over the years were 67.29 and 32.71 percent, respectively.

Table 5: Sales and mortality patterns of crossbred mecheri lambs

Year	Number of animals	Type of sale		Mortality age category	
		Breeding sale (%)	Slaughter sale (%)	Young (%)	Adult (%)
2014	80	0.00 (0/80)	100.00 (80/80)	52.50 (42/80)	47.50 (38/80)
2015	54	62.96 (34/54)	37.04 (20/54)	74.07 (40/54)	25.93 (14/54)
2016	28	42.86 (12/28)	57.14 (16/28)	96.43 (27/28)	3.57 (1/28)
Total	**162**	**28.40 (46/162)**	**71.60 (116/162)**	**67.29 (109/162)**	**32.71 (53/162)**

Source: Authors' own elaboration.

4. Discussion

The overall least-squares means for body weight of crossbred lambs at the different ages were comparable with those observed in the Mecheri purebred sheep population (Thiruvenkadan *et al.*, 2010; Thiruvenkadan *et al.*, 2011; Jeichitra *et al.*, 2015). The body weight of the F_2 generation (progeny of the crossbred ewes) was much lower than the pure Mecheri sheep population at the farm as well as under farmers' field conditions (Ganesakale and Rathnasabapathy, 1973; Acharya, 1982; Jagatheesan *et al.*, 2003; Report, 2004; Karunanithi *et al.*, 2005; Thiruvenkadan *et al.*, 2011; Jeichitra *et al.*, 2014; Jeichitra *et al.*, 2015).

In general, the fixed effects considered in the present study had few significant on body weight and ADG at the different stages of life examined. On the contrary, most of the previous studies in other breeds of sheep reported significant ($p < 0.05$) to highly significant ($p < 0.01$) effects on body weights at the various ages (Mohammadi *et al.*, 2010; Jeichitra *et al.*, 2015). The difference in body weight between male and female lambs with the advancement of age might be due to the increasing differences in the endocrine system between males and females (Swenson and Reece, 1993). These sex differences are consistent with results from other investigations (Mohammadi *et al.*, 2010; Thiruvenkadan *et al.*, 2011). The significant difference in parity of the dam on the birth weight of lambs has also been reported by earlier researchers (Thiruvenkadan *et al.*, 2011; Jeichitra *et al.*, 2015).

The overall pre-weaning ADG observed in this study was comparable with the estimates reported earlier (Thiruvenkadan *et al.*, 2010; Thiruvenkadan *et al.*, 2011; Jeichitra *et al.*, 2015) in the Mecheri sheep breed. The overall least-squares mean indicated that the maximum growth rate had occurred during the pre-weaning stage, and is in agreement with the reports on other sheep breeds (Gowane *et al.*, 2015; Singh *et al.*, 2016; Mahala *et al.*, 2019). This is due to better nourishment of newborn by dam's milk, whereas the post-weaning weight gain depends mostly on grazing, and therefore declines in ADG could be attributed to the competition effect that occurs due to resource sharing between the members of the same flock and grazing stress. Higher estimates for ADG1 and ADG2 have been reported for other sheep breeds of India (Gowane *et al.*, 2015; Singh *et al.*, 2016; Mahala *et al.*, 2019) and in exotic breeds of sheep (Mohammadi *et al.*, 2010; Mokhtari *et al.*, 2013). These differences were due to genetic differences between the breeds and non-genetic differences arising from variation in management and climatic factors. It is seen that Mecheri crossbred lambs attained 72.40 percent of their yearly weight at 6 months of age, and the remaining 27.60 percent of body weight was observed in the later period (ADG3).

Although not addressed in this study, in general the introduction of crossbred Mecheri lambs to the farmer's field conditions revealed the low preference by the local farmers due to the decreased quality of the skin and meat. Hence, the crossbred lambs fetched a lower price when compared to the purebred animals. Under the low-input production system, the survivability of the crossbred lambs was low due to poor birth weight and poorer mothering ability of the ewes due to poor milk yield. As a result, the crossbreeding programme has been discontinued and pure breeding was recommended for the MSRS as well as under farmers' field conditions for further genetic improvement.

5. Conclusions

Selective breeding of the Mecheri sheep with conventional procedures combined with molecular markers has been suggested for faster genetic improvement. The introgression study revealed that there was an increase in twinning rates in crossbred ewes. However, due to lack of interest by the farmers as a result of reduced skin and meat qualities and increased lamb mortality due to poor birth weight of the multiple born lambs led to discontinuation of the project. This result suggests that a thorough analysis of the consumer preference towards rearing of crossbred sheep in their native breeding system is needed before starting any new genetic improvement programme by crossbreeding.

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Allelic variation of Bovine Major Histocompatibility Complex – *DRB3.2* in *Bos indicus cattle* and *Bos indicus* X *Bos taurus* crossbred cattle[1]

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Abstract

The bovine leukocyte antigen *BoLA-DRB3* gene is highly polymorphic and associated with many infectious diseases of dairy cattle. Many studies have thus focused on the correlation between *BoLA-DRB3* and disease resistance. The present study focused on the relationship of *BoLA-DRB3* allelic variants in indigenous as well as in crossbred dairy cattle in India. The number of PCR-RFLP variants and types of DNA sequences indicate the involvement of more than one allele in PCR amplified genomic DNA. The PCR-RFLP results revealed that *BoLA-DRB3.2*8* (9.03 percent) and **23* (10.57 percent) alleles were significantly ($p < 0.05$) more frequent in mastitis susceptible crossbred cattle, whereas in mastitis resistant Indian zebu (Kangayam, Ongole, Deoni and Bargur) breeds of cattle *BoLA* alleles of *DRB3.2*6* and *DRB3.2*15* were prevalent. The results indicate that *BoLA-DRB3.2* allelic variation differs substantially between Indian zebu cattle and crossbred cattle.

Keywords

bovine, MHC, DRB3, BoLA, disease resistance

1. Introduction

The Bovine Major Histocompatibility Complex (*MHC*) (bovine leukocyte antigen-*BoLA*) DR region consists of the monomorphic *BoLA-DRA* locus and three *DRB* loci, of which the fully functional gene is *BoLA-DRB3* (Burke, Stone and Muggli-Cockett, 2009). *BoLA-DRB3* alleles have a significant association with different indicators of infection (Dietz *et al.*, 1997; Nascimento *et al.*, 2006; Kulberg *et al.*, 2007; Juliarena *et al.*, 2008; Nikbakht Brujeni, Ghorbanpour and Esmailnejad, 2016; Carignano *et al.*, 2017) and vaccine responses (Rupp, Hernandez and Mallard, 2007; Gowane *et al.*, 2013). Information on the association of *BoLA–DRB3* gene variants with infectious diseases may help in identifying candidate genes that could then be included in selection programmes. Identifying

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specific alleles that have associations with disease resistance could represent a milestone in developing disease resistant crossbred animals.

Many studies have investigated the genetic association of the *MHC* class *II DRB3* alleles with resistance to mastitis (Dietz *et al.,* 1997; Duangjinda *et al.,* 2013; Othman *et al.,* 2018). The general consensus from studies on exon 2 of *DBR3 (BolA-DRB3.2)* is that these genes are potential genetic markers for higher or lower risk of disease occurrence in cows. The *BoLA* class *II DRB3* allele **16* (*BoLA-DRB3.2*16*) plays a role as a risk factor for higher somatic cell count, which is an indicator of mastitis (Dietz *et al.,* 1997). Further, studies conducted in different states within India have reflected a high incidence of the disease for past several decades. In a recent survey (Joshi and Gokhale, 2006), the occurrence of clinical mastitis in various parts of the country ranged from 11.51 to 23.55 percent, 3.94 to 17.25 percent and 1.99 to 12.28 percent in crossbred cows, indigenous cows and buffaloes, respectively. The corresponding figures for subclinical mastitis varied from 18.40 to 72.60 percent, 15.78 to 81.60 percent and 20.72 to 61.73 percent, respectively for the same classes of livestock. The study also indicated the overall incidence of subclinical mastitis in Holstein-Friesian (HF) crossbred cows to be around 46 percent. Hence, objectives of this current study were to determine the allelic variations of *BoLA –DRB3* gene present in indigenous and crossbred dairy cattle of India, by using restriction fragment length polymorphism (RFLP) and sequence based typing, both of which were based on the polymerase chain reaction (PCR).

2. Materials and methods

DNA isolation

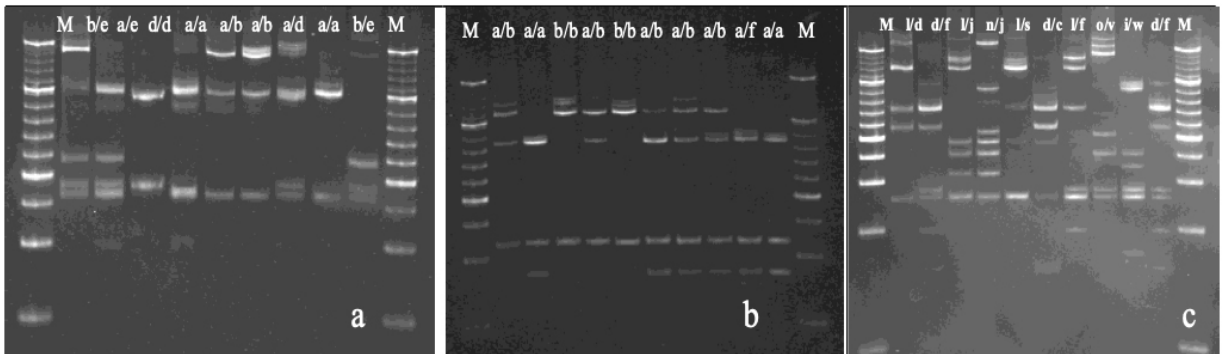
Genomic DNA was extracted from the blood samples of HF crossbred (N = 230) and Jersey crossbred (N = 279) cows (mastitis susceptible breeds) and also from Indian cattle: Kangayam (N = 109); Bargur (N = 75); Ongole (N = 110) and Deoni (N = 91) (mastitis resistant breeds).

DRB3.2 Gene amplification

Isolated genomic DNA was subjected a two-step semi nested (PCR) to amplify the *BoLA-DRB3.2* alleles with certain modifications (Eijk, Stewart-Haynes and Lewin, 2009). A 284 base-pair (bp) fragment of *BoLA DRB3* gene, (comprising 10 bp of the 5′intron, 267 bp of the exon 2 and 7 bp of the 3′intron) was amplified. The first round of PCR reactions was performed using approximately 50 ng of DNA (2 µl) in a final volume of 25 µl containing 5 pmols each of HLO30 (5′- ATC CTC TCT CTG CAG CAC ATT TCC-3′) and HLO31 (5′- TTT AAT TCG CGC TCA CCT CGC CGC-3′) primers, 100 µM dNTPs, 2.5 mM Mg Cl_2, 50 mM KCl, 10 mM Tris-HCl and *Taq* DNA polymerase 1.0 unit. The thermal cycling consisted of initial denaturation at 94 °C for 5 min followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 1 min followed by final extension at 72 °C for 5 min. After the first round, a semi-nested second round PCR reaction was carried out with 1 µl of the first round PCR product as DNA template, containing the same volume and concentrations as described above expect with primers HLO30 (5′- ATC CTC TCT CTG CAG CAC ATT TCC-3′) and HLO32 (HL032 5′- TCG CCG CTG CAC AGT GAA ACT CTC-3′). The thermal profiling consisted of initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 65.5 °C for 30 seconds and extension at 72 °C for 1 min followed by final extension at 72 °C for 5 min.

Restriction Fragment Length Polymorphism (PCR-RFLP)

A PCR-RFLP analysis of the *BoLA-DRB3.2* alleles was carried out to determine different allelic patterns in purebred Kangayam, Bargur, Ongole, and Deoni, and crossbred Jerseys and HF cows. The PCR-RFLP was carried out with three different restriction enzymes viz. *Rsa*I, *Bst*YI and *Hae*III in three cattle breeds separately. Alleles were determined using Molecular Analyst Software (Bio-Rad, Hercules, CA, USA) and shown in Figure 1. The



Source: Authors' own elaboration.

Figure 1: PCR-RFLP patterns of *BoLA-DRB3.2* alleles in Deoni cows using (a) BstYI, (b) HaeIII and (c) RsaI restriction enzyme digestion electrophoresed in 10 percent polyacrylamide gel (M is 50 bp ladder, followed by restriction type to the respective endonuclease enzymes)

BoLA-DRB3 alleles were determined according to the *BoLA* allelic nomenclature (Russell *et al.*, 1997) . The combination of the banding patterns, from digestion with each of the enzymes, determines the genotype of the individual. The nomenclature for alleles of *BoLA-DRB3* defined by the PCR-RFLP method was indicated by the format locus. exon*allele, e.g. *DRB3.2*16*. The same method was followed to determine the *BoLA-DRB3.2* alleles.

PCR Sequence Based Typing (PCR-SBT)

In addition, direct sequencing of *BoLA-DRB3.2* allele was carried out using an automated DNA sequencer (ABI prism) using Sangers's dideoxy chain termination method to confirm the polymorphism at the nucleotide level. Both forward and reverse primers were used for sequencing the PCR products and were custom sequenced (Scigenom, Kerala & Amnion, Bangalore, India). The nucleotide sequences were analysed with Lasergene DNASTAR and BIO edit software (Hall, 1999). The significance of differences in *BoLA-DRB3* allelic frequencies among breeds was evaluated by using the χ^2 test.

3. Results and discussion

The size of the PCR product was 284 base pairs (double stranded DNA marker) yielding different restriction patterns by *Rsa*I, *Bst*YI and *Hae*III restriction enzymes, and all possible allelic combinations were observed. The estimated frequencies of *BoLA-DRB3.2* allele in Deoni, Ongole, Kangayam, Bargur, Jersey and HF crossbred cattle are summarized in Table 1 and the frequency distribution is shown in Figure 2. The most common alleles in HF crossbred and Jersey crossbred cattle were *BoLA-DRB3.2*8* (9.03 percent) and **23* (10.57 percent). Among the major alleles (*BoLA-DRB3.2*8, *10, *13, *15, *16, *22, *23, *24* and **47*), the allele **22* was significantly ($p < 0.05$) more common in crossbred cattle. The *DRB3* alleles of **6, *9, *11, *13, *15*, and **23* were much less frequent (1.08, 1.29, 3.23, 4.95 3.87 and 4.30 percent, respectively) and alleles, **8, *23* and **24* were relatively high in frequency (9.03, 10.57, 7.89 percent, respectively). The common alleles (*BoLA-DRB3.2 *8, *10, *16 *22, *24* and **47*) in crossbred cattle were less frequent in the native animals. In crossbred cows affected with mastitis, the frequency of allele **8* was significantly high ($p < 0.05$) when compared to the healthy crossbred animals. In Kangayam, Ongole, Deoni and Bargur breeds of cattle, alleles **6* (19.72 percent), **6* (24.00 percent), **15* (22.94 percent) and **6* (19.23 percent) had the highest frequency, respectively. Six common alleles (*BoLA-DBB3.2*6, *9, *11, *13, *15* and **23*) combined for more than 50 percent of total allelic frequencies. In this study, these alleles (**6* and **15*) were significantly ($p < 0.05$) higher in low milk yielding native Kangayam, Bargur, Ongole and Deoni breeds of cattle.

Table 1: Allele frequencies of *bola –drb3.2* in indigenous and crossbred cattle breeds

Breed	Allelic frequency			No. of alleles
	< 5%	5 to 10%	>10%	
Kangayam *N* = 109	*BOLA-DRB3.2*1,3,8,12,14,16,20,27,28,36,37,38,41,42,46,N*	9,11,13,23,31,34	6,15[a]	27
Bargur *N* = 75	*BOLA-DRB3.2*1,12,13,14,16,19,20,24,25,27,31,34,35, 36,37,38, 41,46,47,51,54*	9,15,23	6[a],11[a]	25
Ongole *N* = 110	*BOLA-DRB3.2*1,3,8,9,11,14,16,20,24,27,28,32,34, 36,37,42,47*	12,23,31	6[a],13,15[a]	26
Deoni *N* = 91	*BOLA-DRB3.2*1,8,13,14,16,17,19,20,22,24,25,27, 28,31,34,36,37,38,41,46, 47,54*	9,15,23,51	6[a],11[a]	28
HFx *N* = 230	*BOLA-DRB3.2*1,2,3,6,9,11,12,13,14,15,16,17,18,20, 22,23,26,27,28,31,32,34,36,37,38,39,41,46,48,49, 51,eaf,fab,gba,xea,N*	8[a],10,24[a],47	-	41
JX *N* = 279	*BOLA-DRB3.2*1,2,3,6,9,11,12,13,16,17,18,19, 20,21,22,25,26,27,28,31,32,34,36,37,39,41,46, 48,49,50,51,54,gba,N*	13,15,24[a],47	23[a]	40

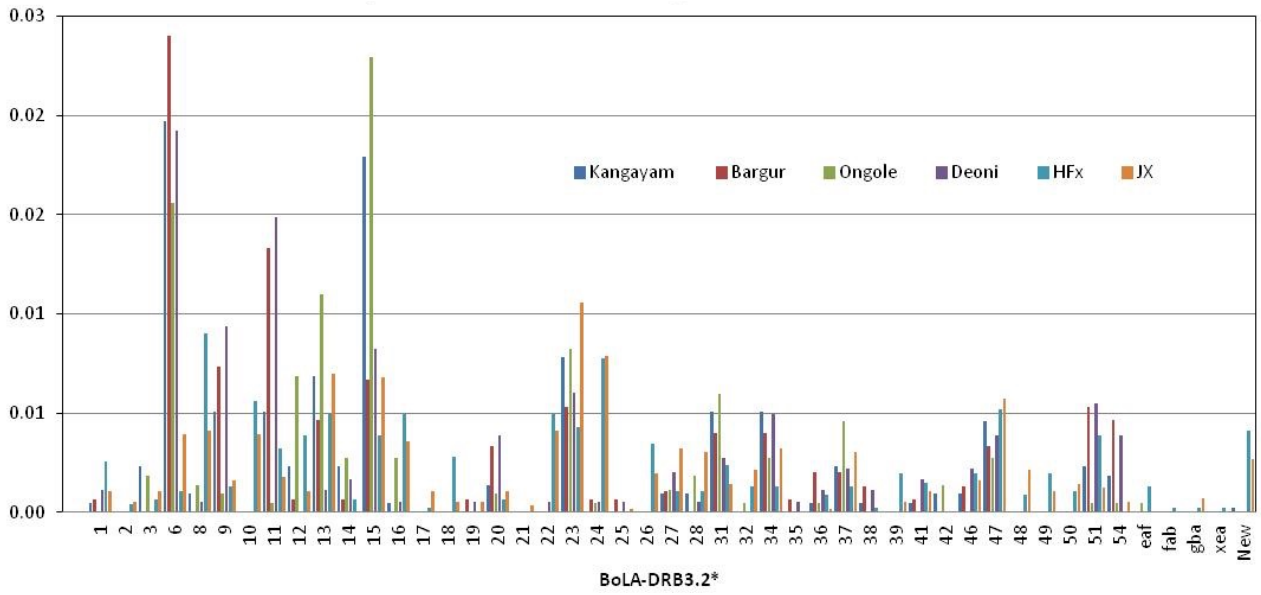
Source: Authors' own elaboration.

Notes: a * Significantly ($P < 0.05$) higher gene frequency based on Chi-square test.
b [a] superscript indicates highest frequent allele pertaining to each breed.
c HFx – Holstein Frisian Crossbred cattle; Jx – Jersey Crossbred Cattle.

Different sequence patterns were observed between *Bos indicus* groups and *Bos indicus x Bos taurus* crossbred genetic groups. All the sequences from six different genetic groups revealed that the *BoLA-DRB3* exon 2 amplified was 284 bp in length. The PCR-SBT analysis of *BoLA-DRB3* exon 2 in all six breeds also confirmed that there are numerous variations in exon 2, which led to different restriction patterns (Table 1). The retrieved sequences of crossbred and *Bos indicus* cattle were compared to reference sequence of the National Centre for Biotechnology Information (NCBI) of the United States of America (Accession No. NC_007324) of MHC class II antigen gene and reflected

the presence of a polymorphic pattern in each sequenced sample.

Allelic variations in *BoLA-DRB3* are unique in many breeds. Similar to the present study, many researchers have found more than 100 different allelic variations around the world by PCR-RFLP methods (Nascimento *et al.,* 2006; Eijk, Stewart-Haynes and Lewin, 2009) and PCR-SBT (Russell *et al.,* 1997). Various alleles have been found to be related with susceptibility and (or) resistance to cattle diseases. The allelic variations observed in crossbred animals are similar to the results observed in other studies. For example, allele *DRB3.2*8* had previously been associated with increased susceptibility, while *DRB3.2*11* and *DRB3.2*23* were associated with increased resistance in HF cattle (Kelm *et al.,* 1997). *BoLA-DRB3.2*16* and **24* alleles were more prevalent in mastitis susceptible animals and *BoLA-DRB3.2*11, *12* and **23* alleles were more prevalent in intramammary infection resistant HF cows (Starkenburg *et al.,* 1997). Forty *BoLA-DRB3* alleles were previously reported in HF x Zebu crossbred dairy cows with frequencies ranging from 0.005 to 0.139 (Duangjinda *et al.,* 2013). In contrast to our study, alleles *DRB3.2*1* and **52* were associated with clinical mastitis, whereas **15, *51,* and **22* were considered as resistant alleles in HF × Zebu crossbred cattle, even though they were raised under tropical conditions (Duangjinda *et al.,* 2013) and the allele *DRB3.2*10* had the highest association with increased milk yield with moderate resistance to clinical mastitis.



Source: Authors' own elaboration.

Figure 2: Allele frequencies of *BolA – DRB3.2* in indigenous and crossbred cattle breeds

Many reports have suggested that the *BoLA* allelic variation differs between *Bos indicus* and *Bos taurus* species and also between breeds. The number of *BoLA DRB3* alleles varied from 19 in Gir to 33 variants in Nellore-Brahman zebu breed (Takeshima *et al.*, 2018). Further, only 14 alleles were shared between *B. indicus* and *B. taurus* animals among the 26 alleles in the IPD-MHC public database (www.ebi.ac.uk/ipd/mhc). These results strongly showed that variation occurs in the *BoLA DRB3* in *B. indicus* and *B. taurus* cattle breeds. The *BoLA-DRB3* polymorphism patterns of Bangladesh, Ethiopian, and Korean cattle are comparable to the present findings (Mandefro *et al.,* 2021). They also confirmed that Zebu cattle breeds had a gene diversity score greater than 0.752, nucleotide diversity score greater than 0.152 and mean number of pair wise differences exceeding 14 while studying the level of genetic variability in the three populations.

In the present study, animal selection was carried out in two different genetic groups, viz. *Bos indicus* Vs *Bos indicus* x *Bos taurus* crossbreds, and these groups differ substantially in their history of mastitis infection, phenotype and production performance even in the same herd. Similarly, the *BoLA – DRB3.2* allelic frequency differed between the species and breeds. Frequency of allele **23* was largest in Jersey crossbred cattle and allele **8* was most frequent in HF crossbred cattle, and both of them are highly susceptible breeds for mastitis infection, based on our anecdotal information. But this allelic frequency was less than 5 percent in all the indigenous Kangayam, Bargur, Ongole and Deoni cattle breeds studied. On the other hand, all the indigenous cattle DNA samples showed that the alleles **6* and **15* were higher in frequency which are very much less frequent in crossbred cattle but not at a zero frequency, since all the crossbred cattle originated from the locally available indigenous breeds. Hence, our present findings elucidate that the higher frequency of *BoLA-DRB3.2*8* and **23* and lower frequency of *BoLA-DBRB3.2*6* and **15* alleles in crossbred cattle could be the reason for the higher mastitis infection in *Bos indicus x Bos taurus* crossbred cattle.

4. Conclusions

The amplified fragment of *BoLA-DRB3.2* alleles was found to be highly polymorphic as revealed by the PCR-RFLP and PCR-SBT variant data. The numbers of PCR-RFLP variants indicate the involvement of more than one allele in PCR amplified genomic DNA. The alleles *DRB3.2*8* and **23* were found to a have higher frequency in mastitis susceptible HF and Jersey crossbred cattle. Meanwhile, the alleles *DRB3.2*6* and **15* had higher frequencies in Kangayam, Ongole, Deoni and Bargur, breeds of cattle that have lesser incidence of mastitis based on our anecdotal data. The allele frequencies were quite different between Zebus and their crossbred, and the increased frequency of *BoLA-DRB3.2*6* and **15* alleles in crossbred population may marginally lower the risk of mastitis infection in that population.

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Breeding for sheep parasite resistance in extensive production systems in Uruguay: From phenotype to genotype

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Abstract

Gastrointestinal parasites (GIP) are one of the main causes of economic losses for sheep farmers worldwide. The need for alternative control measures comes from increasingly critical anthelmintic resistance. One alternative is to include genetic resistance to GIP in breeding programmes, by selecting for worm faecal egg count (FEC). Using this selection criterion since 1994, Uruguay has included genetic resistance to GIP in the genetic evaluation of Australian Merino and Corriedale breeds. Although FEC has been the most used selection criterion to evaluate resistant animals, data recording is time-consuming and costly and requires a nematode infection challenge. Selecting parasite resistance without the need for nematode challenge would be a less expensive alternative approach without compromising the wellbeing of the animals. Moreover, other indicator traits such as packed cell volume (PCV), FAMACHA© score, body condition score (BCS) could be included to increase genetic improvement. This paper describes the current selection programmes for GIP-resistant sheep,

data recording, new criteria evaluation, selection nuclei, development and use of molecular tools, projects, as well as further approaches to enhance and improve genetic progress in Uruguay. Current databases enabled various estimations and demonstrated that genetic progress can be achieved. We can highlight the following results: (i) FEC heritability values ranging from 0.15 to 0.21; (ii) high genetic correlation between FEC in ewes at spring rise and FEC in lambs at post-weaning (0.81 ± 0.11); (iii) genetic selection by FEC is effective in different environments (low or high worm environments) and the genetic correlation between environments is high (0.87 ± 0.04); (iv) there is a moderate favorable genetic correlation between FEC and FAMACHA©; (v) the Corriedale susceptible line had up to 3.3 times higher average of FEC than resistant line; (vi) INIA Corriedales showed better genetic merit for twinning rate, greasy fleece weight, fibre diameter, and body weight at shearing in comparison with the resistant line of SUL; and (vii) in Australian Merino, it has been possible to generate heavier progeny producing more and finer wool, and also more resistant to GIP. Moreover, generating reference populations for molecular studies and selection nuclei is also very important. All strategies described in this study aim at improving the genetic resistance of sheep to GIP.

Keywords

Corriedale, FEC, *Haemonchus contortus*, Merino, SNP

1. Introduction

Worldwide, gastrointestinal (nematodes) parasites (GIP) generate numerous productive and economic losses in sheep production and Uruguay does not escape from this problem. Gastrointestinal parasites inhabit the digestive system and can trigger chronic conditions that jeopardize productive potential (Radostits *et al.,* 2006). A wide variety of domestic animals are affected, and small ruminants are among the most susceptible. Although some mortality is observed, subclinical diseases are frequent, with poorer growth rate, fertility, milk, and wool production and body condition; resulting in large losses for farmers (Castells *et al.,* 1995; Jackson *et al.,* 2009). Due to the serious situation of anthelmintic resistance for all commercial drugs available, the use of non-chemical alternative strategies is essential to address the problem of GIP. For this reason, the inclusion of genetic resistance to GIP as one selection objective in a comprehensive breeding goal is being considered by commercial farmers and stud breeders who raise their sheep in temperate and subtropical areas under extensive production systems. By improving sheep GIP resistance, animal productivity increases (Castells *et al.,* 1995) and the use of anthelminthic drugs can be reduced (Byrne *et al.,* 2012), leading to lower environmental chemical contamination and less drug residue in tissues. Another benefit of selection for GIP resistance is improving animal welfare through more resilient sheep, that are better adapted to the environment (Bishop, de Jong and Gray, 2003).

In Uruguay, since 1994, genetic resistance to GIP has been included in the genetic evaluation systems of the Australian Merino and Corriedale breeds. The selection criterion is faecal worm egg count (FEC), according to a protocol based on the Australian experience (Cardellino, Peñagaricano and Castells, 1994). The FEC is recorded in lambs after weaning in two independent natural challenges separated by an effective anthelminthic treatment. Faecal samples are collected from the animal's rectum and after identification they are sent to the parasitology laboratory for FEC determination within 72 hours. The FEC is determined using a modified McMaster technique (Whitlock, 1948). Under this natural uncontrolled challenge, the most prevalent nematode is *Haemonchus sp.* followed by *Trichostrongylus sp.* (Castells, 2009, Goldberg, Ciappesoni and Aguilar, 2012, own data surveyed in the stud-flocks since 2017). Depending on the epidemiological conditions, the first and second FEC records are made at 8-9 and 10-12 months of age of the lambs (Ciappesoni, Goldberg and Gimeno, 2013). This corresponds to the months from May to June and from July to September, respectively, and thus to the end of the autumn and the winter in Uruguay. The weather conditions in Uruguay are characterized by an average annual temperature of about 17.7 °C and average annual accumulated rainfall between 1 200 and 1 600 mm with a great interannual variability (Castaño *et al.*, 2011). For example, in extreme years, minimum and maximum values are lower than 20 and greater than 250 mm/month respectively (Castaño *et al.,* 2011).

Stud-breeders (supported by the Rural Association of Uruguay) provided genealogical information and performance data recorded at stud level through the SULAR software developed by the Uruguayan Wool Secretariat (SUL). The genetic evaluation is performed by a repeatability animal model adjusting the FEC data through natural logarithms Log_e (FEC + 100) to normalize the residual distribution (Ciappesoni, Goldberg and Gimeno, 2013). Animal genetic merit is published as the expected progeny difference (EPD) for FEC. The genetic trends are presented as estimated breeding values (EBV = EPD × 2). National genetic evaluations (NGE) of wool and meat quality and production traits are routinely carried out by SUL and the National Agricultural Research Institute (INIA) (www.geneticaovina.com.uy).

Although FEC has been the most used method to evaluate the resistance of animals to GIP, there are other indicator traits such as packed cell volume (PCV), FAMACHA© score, and body condition score (BCS),

among others. The protocol for recording these traits is described in Marques, Goldberg and Ciappesoni (2020). All these traits are recorded by INIA trained technicians. To determine the PCV, blood samples collected from the external jugular vein in tubes containing anticoagulant were subjected to hematologic analysis. Values were determined using the routine microhematocrit method reported by Schalm (1986). For the BCS analysis, scores ranged from 1 to 5, where higher numbers indicate increasing amounts of fat cover (Jefferies, 1961).

Selecting for GIP resistance without the need for nematode challenge could be a less expensive and laborious alternative approach that does not compromise the animal wellbeing and may thus lead to greater adoption by stud breeders. The national approach has been to contribute to the selection of genetically resistant animals within integrated management of GIP, in which genetic resistance can play a very important role. However, because it is a difficult trait to record, the adoption of this selection criterion in the main breeding programmes has been low. Hence, different strategies have been pursued at the country level to increase the genetic improvement of resistance to GIP. The aim of the present work is to describe the current selection programmes for GIP-resistant sheep, as well as projects and the main strategies to enhance genetic progress in Uruguay.

All the studies were conducted in accordance with the current Uruguayan laws for the care and management of experimental animals (cnea.gub.uy, law 18611). All applied protocols were approved by INIA Animal Ethics Committee (Approval numbers INIA_2018.2, INIA_2018.3 and INIA.2020.17).

1.1 Supporting data recording and evaluation of new criteria

Given the reluctance of breeders to collect FEC data, different projects are underway to support the expansion of the recording. All projects use the same protocol described above. As an example, we can mention the initiative for the development of recording for Corriedale (2002) and Australian Merino (2004) funded by INIA and the Inter-American Bank. More recently, coordination and registration have been done directly at Corriedale (2018) and Merilin (2020) stud flocks, as part of an INIA project in collaboration with the breed societies. Merilin breed is the first synthetic breed developed in Uruguay crossbreeding between Merino Rambouillet (75 percent) and Lincoln (25 percent) (Merilin, 2022). Additionally, Dohne Merino lambs have been recorded at INIA (2008) and SUL (2015) experimental stations and some commercial stud flocks. Currently, the genetic evaluation of the Australian Merino breed is consolidated and growing for the Corriedale and Dohne Merino breeds. A stud-flock of the Merilin breed began the FEC genetic evaluation with the 2019 progeny.

In the last six years, regardless of the breed, the number of stud flocks presenting FEC data has increased, as has the number of lambs evaluated (Table 1 and Table 2). Among the progeny of 2014, 882 lambs from 9 stud flocks were evaluated, while in 2019, 4 105 lambs from 25 stud flocks were evaluated (Table 1 and Table 2). Collection of data is performed by INIA's technicians (i.e. Corriedale and Merilin breeds) or by stud breeders trained, coordinated and advised by INIA's or SUL's technicians (i.e. Merino breeds). The accuracy of FEC EPD in the first genetic evaluation (lambs with own data) ranges between 0.65 and 0.70.

Table 1: Number of stud-flocks with FEC records by breed per year of birth

Breed	Birth year					
	2014	2015	2016	2017	2018	2019
Corriedale	4	7	7	9	11	15
Australian Merino	4	5	6	6	7	7
Dohne Merino	1	1	1	2	3	3
Total	**9**	**13**	**14**	**17**	**21**	**25**

Source: Authors' own elaboration.

Table 2: Number of lambs with FEC records by breed per year of birth

Breed	Birth year					
	2014	2015	2016	2017	2018	2019
Corriedale	121	1 079	1 301	1 451	1 778	1 634
Australian Merino	726	1 298	1 400	1 397	1 906	2 039
Dohne Merino	35	58	95	285	391	432
Total	**882**	**2 435**	**2 796**	**3 133**	**4 075**	**4 105**

Source: Authors' own elaboration.

With the current databases it has been possible to estimate the heritability of FEC for the main breeds in production systems under natural infections. The methods to estimate genetic parameters and breeding values at the national level are described in detail in the cited papers (Castells, 2009; Goldberg, Ciappesoni and Aguilar, 2012; Ciappesoni, Goldberg and Gimeno, 2013). In brief, a multivariate animal model was used to estimate genetic parameters and breeding values with the BLUPF90 family of programs (Misztal *et al.*, 2018). Variance components were estimated using a Bayesian methodology (GIBBS2F90 software). Heritability estimates range between 0.15 ± 0.01 for Merino (Ciappesoni, Goldberg and Gimeno, 2013) and 0.21 ± 0.02 for Corriedale (Castells, 2009). These estimates are in agreement with international studies (i.e. review by Safari, Fogarty and Gilmour, 2005), which indicate moderate FEC heritability in lambs (0.2 to 0.4), thus enabling genetic progress by selection. The association between nematode resistance in periparturient ewes and post-weaning lambs in Australian Merino sheep was also investigated (Goldberg, Ciappesoni and Aguilar, 2012). Lambing season in the studied flocks was Spring (most births taking place in September and October) and weaning occurs generally between December and January (late Spring, early Summer). Two repeated post-weaning FEC records in lambs were registered in these flocks in June and from August to September for FEC 1 and 2, respectively. These flocks were in the northern part of Uruguay, characterized by warm and wet climate, with a mean annual temperature of 18–19 °C, relative humidity of 70–72 percent and an average annual rainfall of 1 400 – 1 500 mm (Castaño *et al.*, 2011). Animals are kept outdoors all year around and graze mostly native grasslands and have access to improved pastures or are supplemented with grains or commercial rations when nutrient requirements are high (e.g. twin bearing ewes) or when native grasslands growth is low (late Winter, dry Summer). This study found a high genetic correlation (0.81 ± 0.11) between the two traits: FEC at spring rise in ewes and FEC in lambs at post-weaning. In addition, this study found that selection based on lamb FEC is twice as efficient as selection based on ewe FEC (Goldberg, Ciappesoni and Aguilar, 2012). In other words, using lamb FEC for indirect selection will be more effective than direct selection on ewe FEC, producing ewes that eliminate less worm eggs resulting in less pasture contamination. Currently, the study of the spring rise phenomenon continues in different breeds and others grazing production systems (Del Pino *et al.*, 2019).

Furthermore, different studies have been carried out looking for new, easy-to-measure, or complementary selection criteria such as FAMACHA© (Ciappesoni and Goldberg, 2018), faecal occult blood test (FOB) (Rodríguez *et al.*, 2015), control of IgA levels (Escribano *et al.*, 2019) and Dag score (RUMIAR project, INIA). Descriptive statistics for traits related to GIP of the total data included in the national database (SULAR) used for the NGE of INIA-SUL (lambs born from 1994 to 2019) are shown in Table 3.

Table 3: Descriptive statistics for traits related to GIP resistance included in the national database (SULAR) from progeny 1994 to 2019

Breed	Trait	*n*	Mean	Standard deviation
Corriedale	BCS at FEC (score)	4 901	3.27	0.76
	FAMACHA (score)	6 577	2.14	0.74
	FEC (count)	25 183	1 525	2 467
	PCV (%)	2 494	35.27	4.75
Australian Merino	BCS at FEC (score)	853	2.85	0.39
	FAMACHA (score)	2 213	2.13	0.86
	FEC (count)	29 645	1 295	1 948
	PCV (%)	1 356	32.05	5.41
Dohne Merino	BCS at FEC (score)	867	3.19	0.47
	FAMACHA (score)	1 171	1.91	0.73
	FEC (count)	1 684	1 314	1 688
	PCV (%)	221	33.85	4.71

Source: Authors' own elaboration.

Note: **FEC: post-weaning Faecal Egg Count. PCV: Packed cell volume. BCS: Body condition score.*

Marques, Goldberg and Ciappesoni (2020) estimated a moderate favourable genetic correlation between FEC and FAMACHA©. In addition, the genetic correlation between FEC measured at low and high worm environments was high (0.87 ± 0.04). This indicates that genetic selection by FEC is effective in different environments. Research is also being carried out to understand the trade-offs of the selection by FEC on other economically relevant traits. The first studies indicate that breeding resistant animals for GIP may not present negative effects on intake or feed conversion efficiency regardless of the level of parasitism (Ferreira *et al.*, 2021). This study evaluated the differences

in feed conversion ratio and residual feed intake in 1-year old lambs (fed ad libitum with a high-protein forage diet) of Corriedale divergent selection lines (see section 2.1).

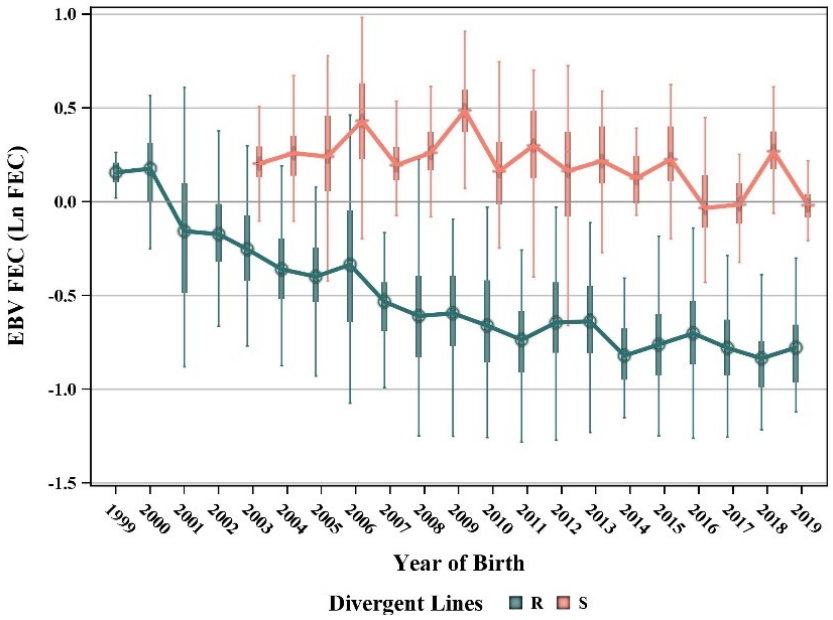
2. Genetic resistance to GIP in selection nucleus

As a strategy for the generation of genetically resistant breeding stock and the dissemination of genetic tools (i.e. EPD), selection nuclei are in place in experimental stations: (1) Corriedale: divergent selection lines by FEC EPD; (2) Australian Merino: selection by FEC EPD and production; (3) Corriedale and Australian Merino: selection by production and FEC EPD.

2.1 Corriedale: divergent selection lines

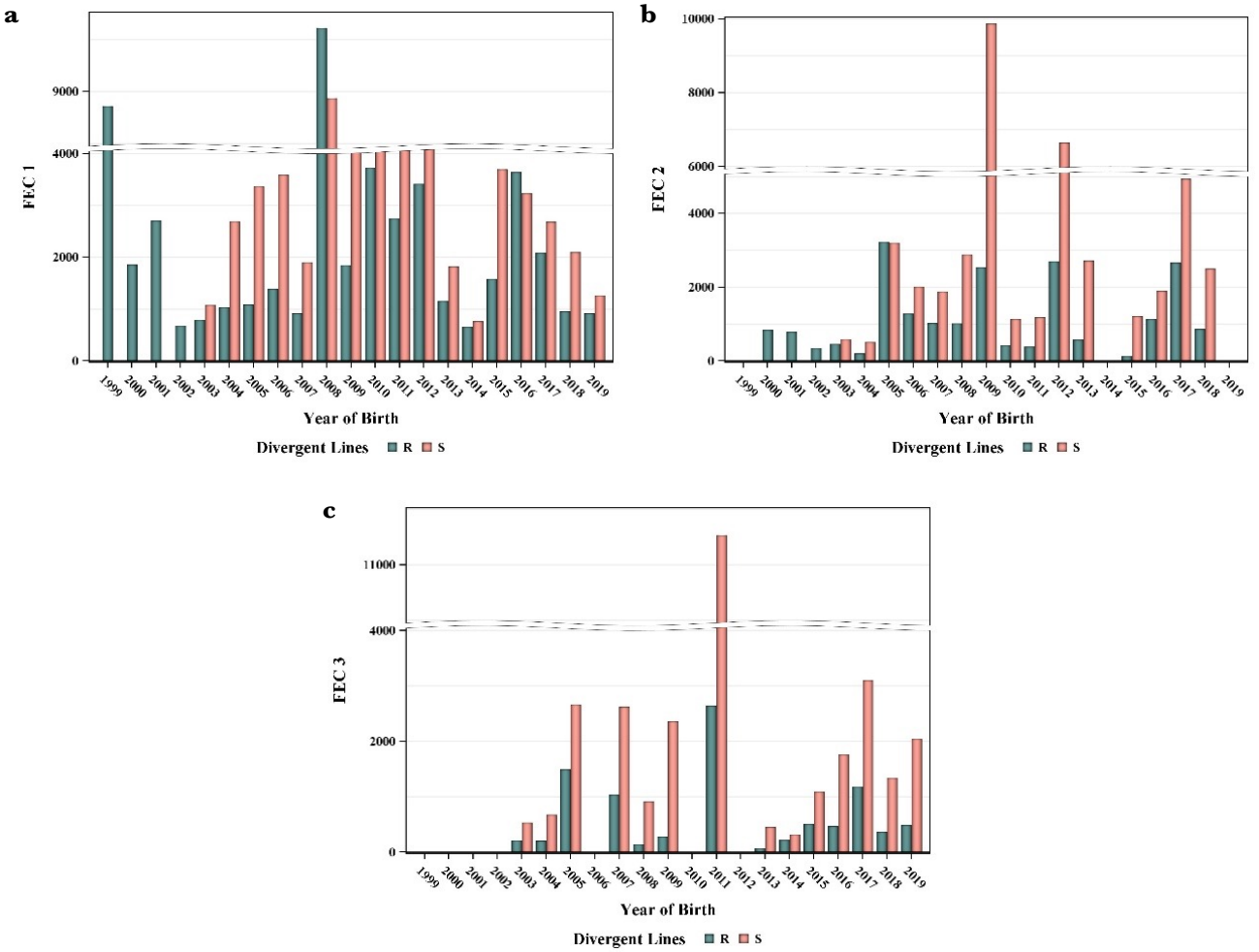
Corriedale divergent breeding lines ewes were selected for low (Resistant line, R-Line) or high (Susceptible line, S-Line) FEC after natural mixed infection and grazing native pastures at *Dr Alejandro Gallinal* Experimental Research Centre of SUL since 1998 (Castells and Gimeno, 2011). After screening for low or high FEC EPD of 3 545 progeny lambs, the nucleus flock was established. More than 150 ewes were annually mated in the R-Line and 120 ewes in the S-Line, and at least five sires were used in each line per mating. Three FEC measurements (FEC1, FEC2, and FEC3) were recorded post-weaning and after natural nematode challenge of different cycles: summer (between 21 December and 20 March), autumn (between 20 March and 21 June) and winter (between 21 June and 22 September). Post-weaning and production traits and complete pedigree were also recorded each year.

The genetic trend of the S-line shows a small reduction. From 2014 progeny, this line has become more like a control line due to the complexity of maintaining a high number of susceptible animals (low longevity or stayability) and because of animal welfare issues. The average number of lambs registered per year were 40 and 80 for the S-Lines and R-Lines, respectively. Additionally, for R-Line some rams from stud-flocks were used. The difference in average breeding values between the first (2000) and last generation (2019) of the R-line represented genetic progress of 15 percent (Figure 1), indicating annual genetic progress of -0.71 percent per year. When analysing R-line FEC average breeding values of the progeny 2019 in comparison with the rest of the Corriedale population in 2019, a genetic difference of 11 percent was observed. This distance between the R-line and the rest of the Corriedale population would be between 5 to 10 years of selection. Considering means of the three records FEC1, FEC2, and FEC3 (at 210 ± 33, 298 ± 43 and 391 ± 53 days of age respectively) for the last five years (from 2015 to 2019 drop) the values for the S-Line were 1.31, 2.47, and 3.26 times higher than the R-Line, respectively (Figure 2). Marked differences were observed between years depending on climatic and epidemiological conditions. Additionally, those phenotypic responses were also observed on lactating ewes during the spring rise 73 days after lambing. The FEC means of R-Line ewes was 395, and 660 for the S-Line (Castells and Gimeno, 2011). It is concluded that by selecting for decreased FEC EPD it is possible to achieve considerable progress, which is reflected, in addition to the genetic trends, in lower infestations in lambs and ewes at spring rise. This experimental flock represents a valuable demonstration population where breeders can see the genetics in practice.



Source: Authors' own elaboration.

Figure 1: Genetic trends of FEC EBV in Corriedale divergent lines (Resistant -R and Susceptible -S) from the Uruguayan Wool Secretariat



Source: Authors' own elaboration.

Figure 2: Phenotypic average for Faecal Egg Count (FEC) 1 (a), 2 (b), and 3 (c) of Resistant (R) and Susceptible (S) lines from 1999 to 2019 progeny from the Uruguayan Wool Secretariat

2.2 Australian Merino: selection by FEC EPD and production

Breeders are reluctant to include genetic resistance to GIP in their breeding programmes because recording FEC is complex and labour-intensive. In 2015, an Australian Merino flock was established at the *Estación Experimental Facultad de Agronomía Salto* (EEFAS, *Universidad de la República*) to intensively select sheep for GIP resistance and thus give access to farmers to high rams with genetic merit for GIP resistance (FEC EPD) without the need for data collection. The selection flock at EEFAS is also a research platform for quantitative and molecular genetics in collaboration with INIA and SUL. The first years of selection were funded by the INNOVAGRO research project (described in acknowledgements) and semen from Australian studs renowned for their resistance to GIP was purchased. High selection pressure on FEC has been applied to the flock resulting in fast genetic progress-producing animals ranking in the top 1 percent of the Australian Merino population in Uruguay in this trait.

The foundation ewes for the GIP resistance flock came from the existing flock at EEFAS which was complemented by ewes from three regional Australian Merino studs (*La Magdalena, Los Arrayanes and Los Manantiales*). In 2015 and 2016, semen from *Rylington Merino* (WA, Australia) was used. The *Rylington Merino* flock was founded in 1988 as an experimental flock by wool farmers and agricultural institutions, firstly by selecting sheep on phenotypic data and from 1994 on by estimated breeding values and became highly resistant to worms (Karlsson and Greeff, 2006). *Rylington Merino* sires have been widely used in flocks participating in Sheep Genetics Australia (www.sheepgenetics.org.au). From 2017 to 2019, semen from the commercial stud *Anderson Rams* (www.andersonrams.com.au/index.html), also located in Western Australia, was utilized. In collaboration with the *Talitas* stud, rams from this stud were used either in a timed artificial insemination programme or in single sire matings with allocated ewes. *Talitas* has been selecting sheep for GIP resistance for more than two decades and produces high-ranking sires in the Uruguayan Genetic Evaluation of Australian Merino. Furthermore, through collaboration with the *Consorcio Regional de Innovación en Lana Ultrafina* (CRILU, Regional Consortium for Innovation in Ultrafine Wool), rams selected for reduced fibre diameter were obtained. Other rams were selected directly from the EEFAS flock based

on their FEC EPD. Table 4 describes the contribution sires and progeny from these studs the FEC EPD data for the animals. Procedures followed to estimate FEC EPD and management practices, and statistical analyses are similar to those previously indicated.

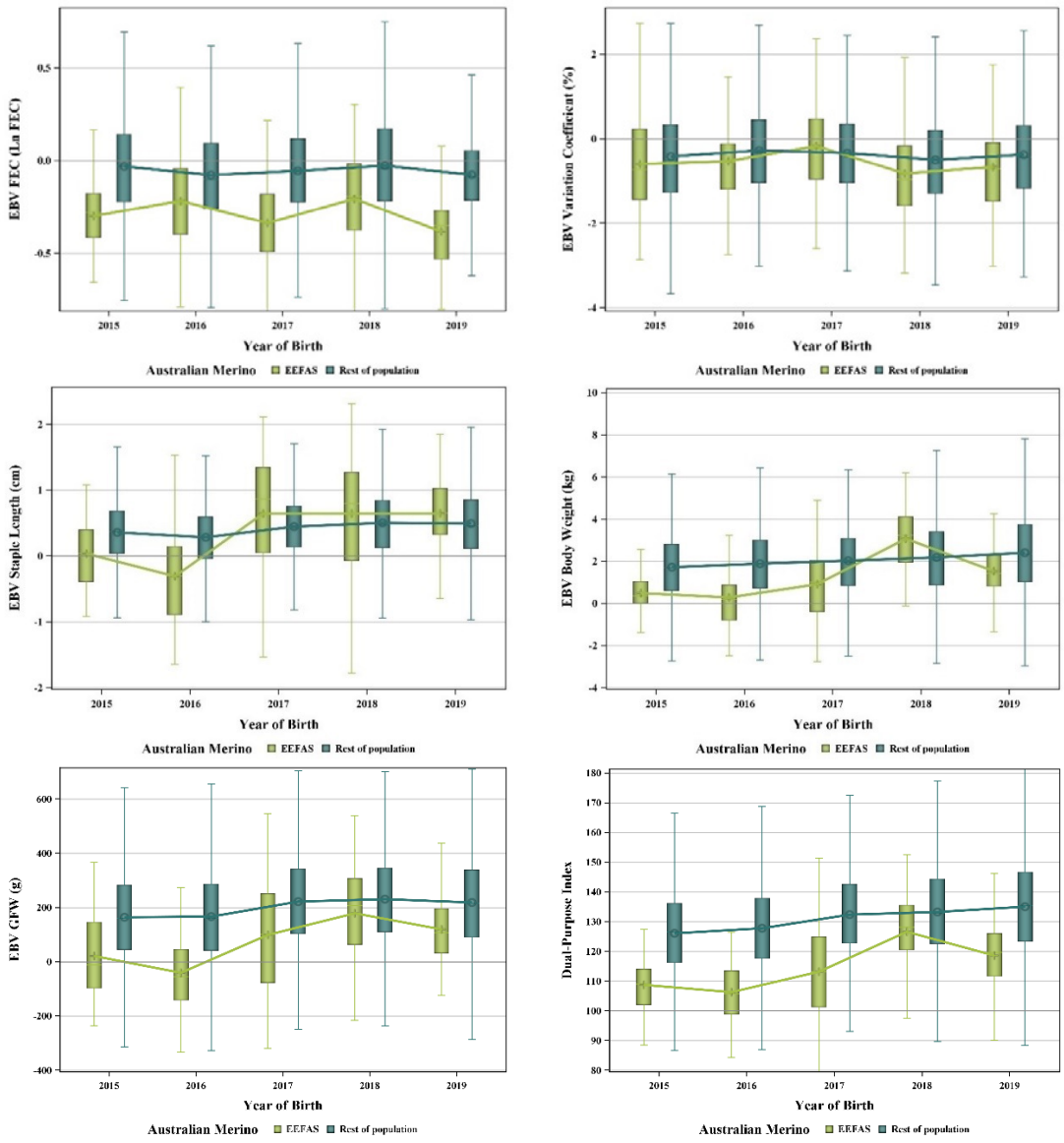
From the beginning, the main breeding goal has been to rapidly increase GIP resistance (reducing FEC EPD). This activity has been a success since the genetic trend for this trait is well below the population trend (Figure 3). One sire from *Rylington Merino*, used in 2015, has produced

Table 4: Number of sires, progeny and expected progeny difference (EPD) of faecal egg count (FEC) data for the studs contributing to the EEFAS flock from 2015 to 2019

Stud	Rylington	Anderson	Talitas	CRILU	EEFAS
Sires (*n*)	3	4	4	7	7
Progeny (*n*)	31	174	62	159	111
FEC EPD *					
Mean	-0.16	-0.11	-0.16	-0.12	-0.20
Std Dev	0.17	0.11	0.10	0.10	0.12
Minimum	-0.45	-0.45	-0.48	-0.39	-0.43
Maximum	0.20	0.15	0.02	0.10	0.25

Source: Authors' own elaboration.

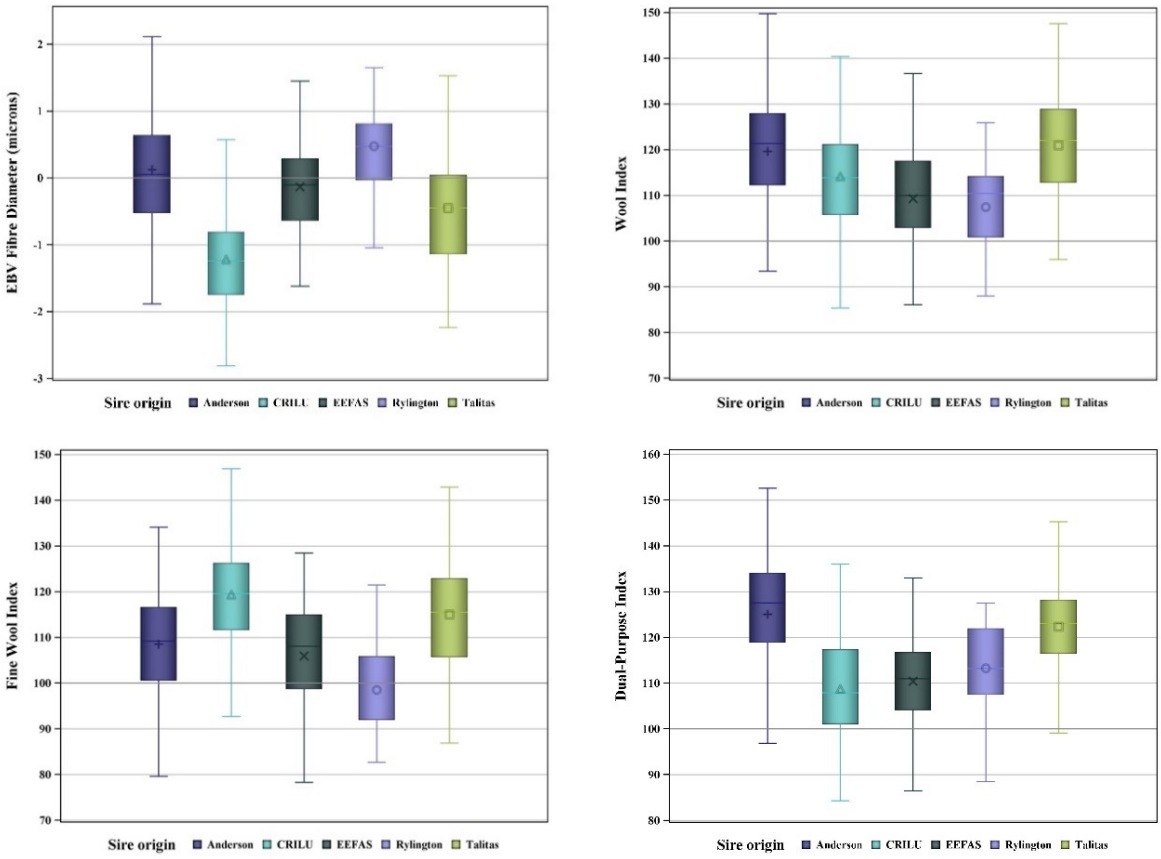
Note: * FEC EPD data according to the 2020 genetic evaluation results (max = -0.54; 1% = -0.30; 5% = -0.20; 10% = -0.15).



Source: Authors' own elaboration.

Figure 3: Five-year genetic trends for Faecal Egg Count (FEC), Variation Coefficient of Fibre Diameter, Staple Length, and Body Weight, Greasy Fleece Weight (GFW) and Dual-Purpose Index of the EEFAS flock and the general Australian Merino population according to the 2020 National Genetic Evaluation; the 2020 drop is not included in the evaluation

the top 1 percent ranking ewes from which several of the EEFAS sires were bred, leaving a long-lasting effect on GIP resistance in the flock. The combination of origins of *Rylington Merino* with *Talitas* has been particularly powerful in delivering top 1 percent ranking EEFAS sires for GIP resistance. Other traits that have improved over time, in comparison with the general population, are the variation coefficient of fibre diameter, staple length, and body weight (Figure 3). The selected sires from *Anderson Rams* have highly contributed to staple length and body weight, as well as greasy and clean fleece weight, while *Talitas* and CRILU sires used have contributed to the reduction of fibre diameter (Figure 4). The genetic evaluation of Australian Merino in Uruguay includes three selection indexes to aid producers in deciding which sire to purchase according to their breeding or production goals; focus on reducing fibre diameter and moderate increases in fleece and body weight (Fine Wool Index), focus on increasing fleece and body weight with moderate reductions on fibre diameter (Wool Index) or a strong focus on increasing fleece and body weight with low reductions in fibre diameter (Dual-Purpose Index) (https://geneticaovina.com.uy/catalogo/catalogo_merino.pdf). Although, the EEFAS flock has not reached top ranks in greasy fleece weight EPD or selection indexes, progress has been made (Figure 3). The step forward is to keep on improving the overall wool quality traits of the progeny and to disseminate correct sires with high genetic merit for GIP resistance.



Source: Authors' own elaboration.

Figure 4: Estimated breeding values for the progeny according to the origin of their sires for Fibre Diameter, Wool Index, Fine Wool Index, and Dual-Purpose Index, based on the 2020 National Genetic Evaluation

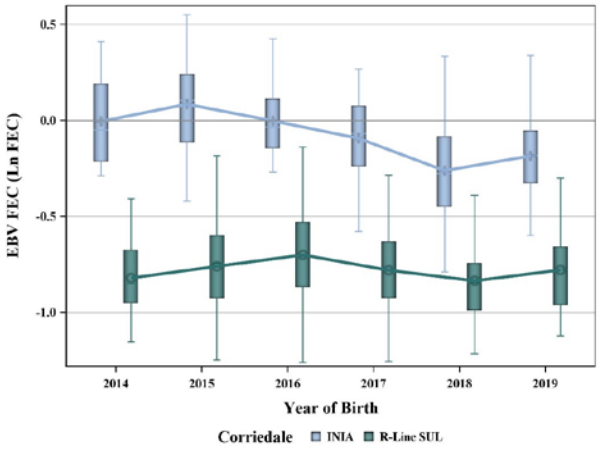
2.3 Corriedale and Australian Merino: selection by production and FEC EPD

The development of the Corriedale nucleus began in 2014 at the Experimental Unit *Glencoe* of INIA to explore the productive and reproductive potential of the breed in extensive grazing conditions. The first ewes came from the Progeny Test Centre *Dr Pedro A. Narbondo.* They were selected mainly by the twining rate EPD and the A-selection index of the breed, which aims to genetically decrease the fibre diameter and increase the clean fleece weight and body weight. The FEC EPD has been just recently included in the breeding programme.

Progeny born between 2014 and 2016 came from 120 original ewes and 17 hoggets born in Glencoe. This nucleus is genetically connected with the Corriedale stud-flocks in NGE. Every year connection rams are used from elite Corriedale stud-flocks. The objective is to search for a balanced flock, in terms of health, reproductive and productive aspects. In the last three years, the reproductive performance of the nucleus, grazing native grasslands, has been excellent for Uruguay, obtaining values of 134 percent lambing (lambs born/ewes joined), 93 percent survival (lambs marked/lambs born) to marking and 120 percent for weaning rate (lambs weaned per ewe joined).

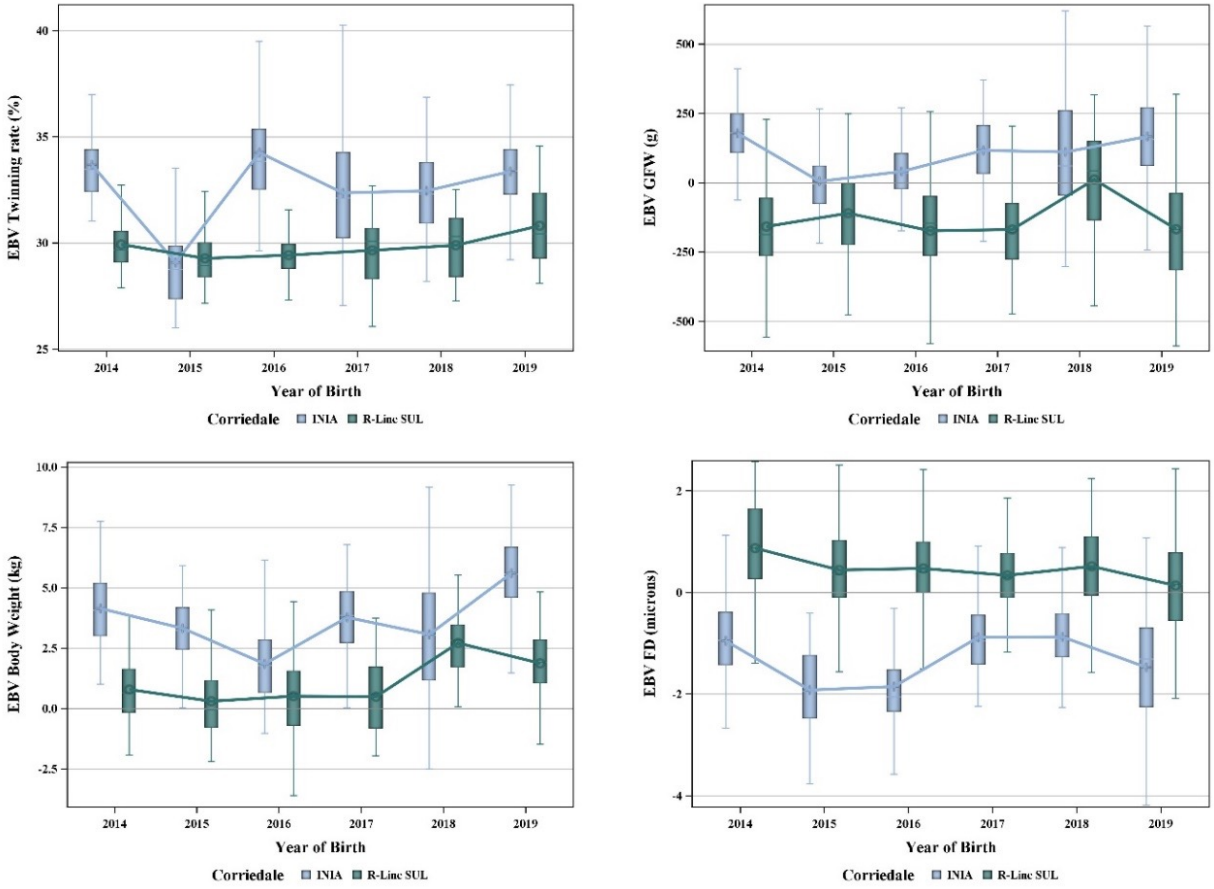
For experimental purposes, in the last two years (from 2019 to 2021), resistant and average rams (negative and close to zero FEC EPD, respectively) for the FEC EPD trait have been used. This contrast is carried out to evaluate the effect of genetic resistance to GIP on other traits such as feed intake, residual feed intake and methane emissions.

When comparing the genetic trends for FEC EBV between the INIA Corriedale nucleus and the SUL resistant line (mentioned in section 2.1), lambs from the later are markedly more resistant (FEC EBV more negative) than the Corriedale of INIA (Figure 5) due to a stronger selection for this trait at SUL. In contrast, INIA animals showed better genetic merit for reproductive and productive traits that are included in the productivity selection indices (Figure 6).



Source: Authors' own elaboration.

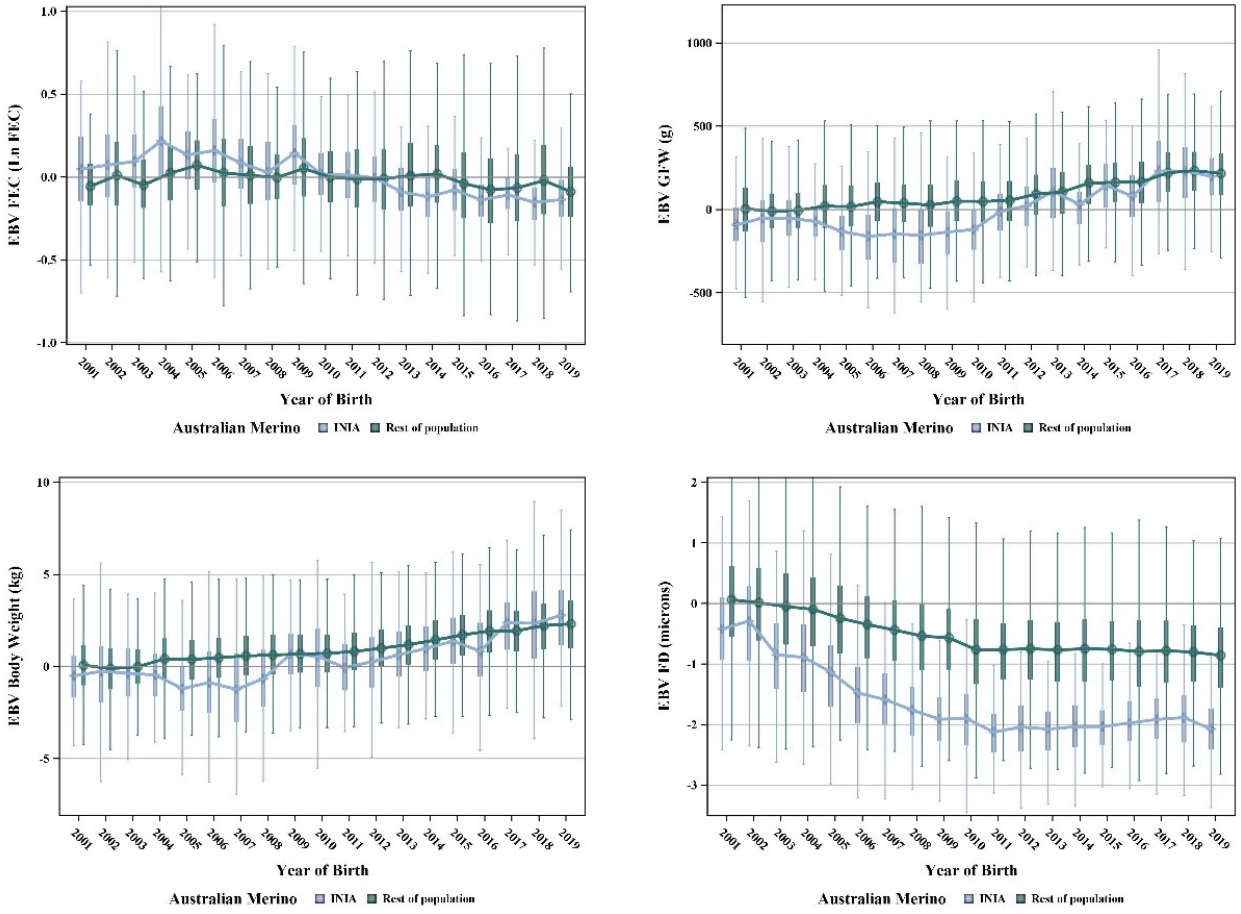
Figure 5: Genetic trends (EBV) for Faecal Egg Count (FEC) of the INIA Corriedale flock in comparison with the resistant line of the Uruguayan Wool Secretariat



Source: Authors' own elaboration.

Figure 6: Genetic trends (EBV) for productive and reproductive traits: twinning rate, greasy fleece weight (GFW), fibre diameter (FD), and body weight at shearing of the INIA Corriedale flock in comparison with the resistant line of SUL

An Australian Merino nucleus was created at the Glencoe Experimental Unit of INIA in 1999. This nucleus was part of the Fine Merino Project of Uruguay (from 1999 to 2010) and CRILU (from 2011 until present) as described by Ramos *et al.* (2021). The main objective of this nucleus has been to identify and generate genetically superior animals and then disseminate them among commercial farmers and stud breeders of Uruguay. Since 1999 the main selection objective has been to produce super- to ultrafine wool without compromising other economically relevant traits such as fleece and body weight, FEC, staple length, wool colour, conformation, and coefficient of variation of fibre diameter, among others. Therefore, the focus during the first years of the nucleus was to drastically reduce fibre diameter (Figure 7). Once wool diameter was in the range of super- to ultrafine wool (2011), the emphasis of selection switched to fleece and body weights and then to FEC. By using the genetic tools available (EPDs), pedigree information, phenotypic records, controlled mating, and visual assessment of the animals, it has been possible to generate progeny that produce more and finer wool, are heavier and more resistant to GIP than previous generations of animals in the nucleus and when compared with the average of the evaluated Merino population (Figure 7). Finally, as mentioned before, the dissemination of superior genetics was an objective. In this sense, from 2000 to 2020, 1 000 superior rams and more than 20 000 doses of semen have been disseminated among farmers and breeders. Thus, it can be considered that the superior genetics generated have been spread across the Merino population of Uruguay.



Source: Authors' own elaboration.

Figure 7: Genetic trends (EBV) for faecal egg count (FEC), fibre diameter (FD), greasy fleece weight (GFW), and bodyweight (BW) at shearing of the Glencoe Merino nucleus in comparison with the rest of the evaluated Merino population in Uruguay

3. Development and use of molecular tools

Initially, many countries aimed to use genetic markers to identify alleles associated with resistance to GIP and to select for breeding young animals carrying these alleles. Marker association studies were carried out in Uruguay using microsatellite molecular markers (STR) in Corriedale (Nicolini, 2006) and Australian Merino breeds (Ciappesoni *et al.*, 2010) and paternity tests were performed in the Corriedale breed (Peraza *et al.*, 2013). Recently, with the development of technologies for data storage and processing and lower costs of equipment and reagents, single nucleotide polymorphism (SNP)

markers have generally outperformed microsatellites for genomic analysis and breeding applications worldwide. A low-density panel of 507 SNP, which includes a subset of markers shared between commercial platforms, was developed in Uruguay (Macedo *et al.*, 2014; Ciappesoni *et al.*, 2016). This panel allows breed genetic identification among populations, paternity assignment (Macedo *et al.*, 2014) and includes markers associated with GIP resistance identified in Australian Merino and Corriedale to improve the prediction of breeding values for genetic resistance to GIP (Grasso *et al.*, 2014).

Nowadays, feasible genomic selection is the preferred approach for genetic improvement, particularly for expensive or difficult to measure traits such as FEC. By using SNP information provided by low/high-density panels, in combination with phenotypic and pedigree information, it is possible to increase EPD accuracy in young animals. Several countries are implementing genomic selection in sheep, such as New Zealand (Auvray *et al.*, 2014), Australia (Daetwyler *et al.*, 2010), and France (Duchemin *et al.*, 2012). In Uruguay, particularly in the Australian Merino breed, progress has been made in the design of the reference population., As of mid-2021 the reference included 2 231 animals with FEC phenotypes and genotypes with either 50K (85 percent) and 15K (15 percent) panels. The first results of genomic evaluation have already been obtained, with the aim of publishing the first FEC the genomic EPD in 2021.

Genomic data provided by SNP panels with various densities have been used for breed genetic characterization and population structure analysis. Several studies considering different breeds or origins have been carried out, such as the genomic comparison of Australian Merino, Corriedale and Creole breeds (Grasso *et al.*, 2014), and Australian Merino with other related Merino breeds (Ceccobelli *et al.*, 2019; Vera *et al.*, 2019). Additionally, metabolic mechanisms and pathways involved in genetic resistance to GIP in the Corriedale breed were identified by using RNA sequencing technology in several tissues (Peraza *et al.*, 2016). Genomic regions associated with FEC have also been identified in the Australian Merino breed in chromosomes 6, 11, 12, and 21, which are consistent with international studies in other sheep breeds (Benavides *et al.*, 2015). In the near future, we plan to undertake selection signature or genome-wide association studies in the Corriedale breed, based on the divergent selection lines by FEC to identify new regions associated with this trait.

INIA's DNA Genomic Bank supports all these research initiatives, with more than 18 000 DNA samples stored from Australian Merino, Corriedale, Dohne Merino, and Creole breeds (Carracelas *et al.*, 2019). From these, more than 6 700 animals have been genotyped with different SNP arrays (Table 5).

Table 5: DNA samples stored at INIA's DNA genomic bank, total number of genotyped samples and samples genotyped by panel

Breed	DNA samples	Genotyped samples	IAEA[1]	UY panel[2]	50K panel[3]	Other panel[4]
Australian Merino	9 597	4 198	0	1 866	1 896	436
Corriedale	6 061	2 369	499	1 446	388	36
Dohne Merino	1 726	0	0	0	0	0
Creole	680	199	0	0	10	189

Source: Authors' own elaboration.
Note: [1]170 SNP; [2]507 SNP UY panel; [3]50K panels included: Affymetrix 54K, Illumina 50K and GGP50K; [4]Other panels included: 15K, 60K and 700K SNP.

4. Conclusions

Genetic resistance to GIP is a high relevance trait to stud breeders and commercial farmers. However, to implement a registration system that allows genetic evaluations, initial support from the institutions is needed (i.e. developing protocols, recording support, training, etc.). Several strategies have been developed to improve sheep genetic resistance to GIP, and further research is ongoing to increase their effectiveness and contribute to greater economic benefits and more sustainable production systems.

Thus, inter-institutional (academy, research, technology transfer) and breeder association's coordinated work and commitment are crucial. In addition, the generation of selection nuclei in experimental stations can play a very important role as demonstration centers, besides the genetic contribution to the commercial flocks. Researchers and institutions need to be up-to-date in the latest procedures such as molecular techniques, in order to provide the best selection tools. Therefore, training people at the local level is very important to achieve this task.

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Genome-wide association study of trypanosome prevalence in Baoulé cattle of Burkina Faso

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Abstract

Trypanosomiasis is a common disease in sub-Saharan Africa caused by the parasitic protozoa of the genus Trypanosoma that affects many species including cattle and humans. The West African shorthorn breed Baoulé is renowned for its ability to withstand trypanosome infection and often remains productive under the disease condition. It is referred to as a trypanotolerant breed. The trypanosomosis status (positive and negative) was recorded for 387 pure Baoulé, animals from the southwest of Burkina Faso, using indirect ELISA. Genotypic data from the

Illumina Bovine SNP50 BeadChip were available for these animals. After applying the quality control criteria, 34 346 SNPs and 343 animals were left for analysis. Single-SNP associations were performed using GEMMA software. An analysis of the data identified six regions on chromosomes 8, 9, 16, 22, 24 that might harbour gene(s) that contribute to the trypanosome resistance in Baoulé cattle.

Keywords

GWAS, trypanosome resistance, candidate gene, cattle

1. Introduction

The sequence of the bovine (*Bos taurus*) genome has been available for some time now (The Bovine Genome Sequencing and Analysis Consortium *et al*, 2009). With the advent of high throughput genotyping technologies, the discovery of single nucleotide polymorphisms (SNPs) and the development of commercial cattle SNP chips, the genotyping of thousands of polymorphic markers have become straightforward. SNP based genome wide association studies (GWAS) can identify chromosome regions that harbour the gene(s) that contribute to the phenotypic variation of a trait or disease like trypanosomiasis, which then could serve as putative regions for further studies (Sahana *et al.*, 2010). Trypanosomiasis is a common disease in sub-Saharan Africa caused by the parasitic protozoa of the genus Trypanosoma that affects many species including cattle and humans. In Africa, it is transmitted by the tsetse fly of the Glossina genus (Courtin *et al.*, 2008, Lamy *et al.*, 2012). Trypanosomes affecting livestock in Africa include *T. congolense*, *T. evansi*, and *T. brucei*, all of which infect blood and tissues. Major symptoms include hyperthermia, anaemia, rapid weight loss, mucous pallor, miscarriage, "petering out", pica, splenomegaly, cachexia, and death (Naessens 2006; Courtin *et al.*, 2008).

In this study, we focus on the discovery of genomic regions affecting trypanosomosis in Baoulé cattle of Burkina Faso. The Baoulé cattle have existed in tsetse challenged zones for hundreds of years and have acquired an immunology phenomenon (trypanotolerance) that has a genetic basis. These animals have a capacity to rid themselves of trypanosome parasites and maintain low parasitaemia (Naessens *et al*, 2002; Agyemang, 2004). Silbermayr *et al.* (2013) found that Baoulé cattle and Baoulé x Zebu crosses in the region have substantially smaller trypanosome infection rates compared to pure local Zebu cattle.

2. Materials and methods

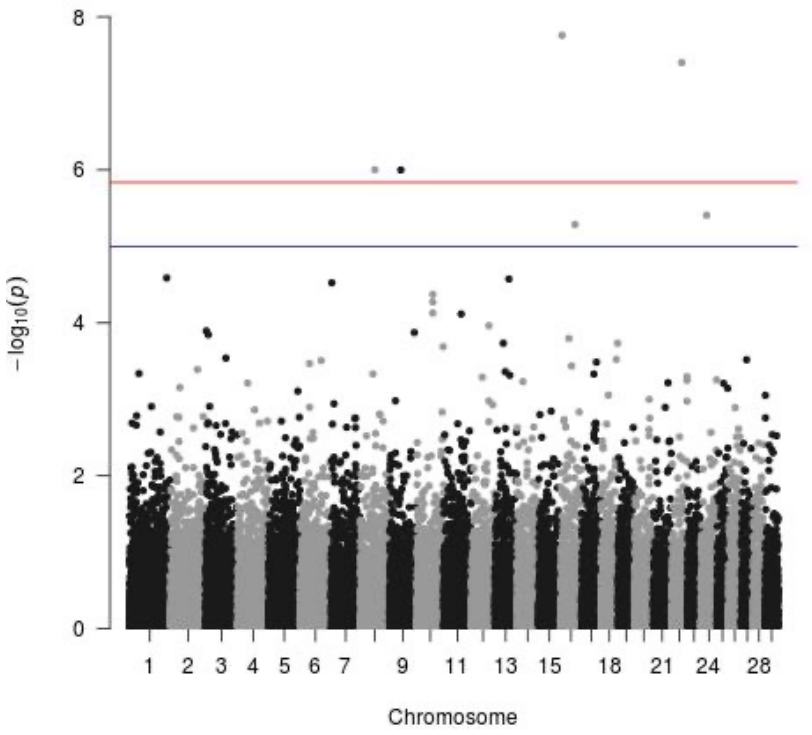
The trypanosomosis status (positive and negative) was recorded for 387 pure Baoulé animals (155 males and 232 females) from the southwest of Burkina Faso, using indirect ELISA (Desquesnes, Bengaly and Dia, 2003). Three sites were selected depending on the availability of Baoulé cattle. In the Bouroum-Bouroum department we worked with sedentary farmers with Baoulé cattle. In the Kampti department we worked with Fulani transhumant herders and Mossi farmers, and in the Loropeni department with sedentary farmers. A total of 88 farms: 55 in Bouroum-Bouroum, 15 in Loropéni and 18 in Kampti were chosen according to the willingness of the farmers to participate in the breeding programme of the Characterization and Sustainable Utilization of Local Cattle Breeds (LoCaBreed) project (https://appear.at/en/projects/current-projects/project-websites/project120-locabreed). The average age of the cattle was 3.76 years. The genotyping of the Illumina Bovine SNP50 BeadChip was performed at Neogen (Lincoln, United States of America) and the data were available for these animals. Quality control of the data was performed with PLINK 1.9 (Chang *et al.*, 2015). The dataset was cleaned using standard quality control to exclude non-autosomal SNPs as well as SNPs with minor allele frequency (< 0.01), those with a call rate of less than 95 percent and those that deviated from Hardy Weinberg equilibrium according to Fisher's exact test with *P*-value of 10E-6. After applying the quality control criteria, 34 346 SNPs and 343 animals were left for the analysis.

Single-SNP associations were performed using GEMMA software (Zhou and Stephens, 2012). GEMMA implements the Genome-wide Efficient Mixed Model Association algorithm for a standard linear mixed model and some of its close relatives for GWAS. It fits a univariate linear mixed model for marker association tests with a single phenotype to account for population stratification and sample structure.

3. Results and discussion

In the single-SNP analyses, 6 SNPs showed significant (-log10 *p*-value = 5) associations with trypanosomosis status (see Figure 1) on chromosomes (CHR) 8, 9, 16, 22, and 24, four of which were significant after Bonferroni correction (p-value < 1.45×10^{-6}), located on CHR16, CHR22, CHR8 and CHR9 (see Table 1).

These results identify chromosome regions that might harbour the genes that contribute to the trypanosome prevalence in Baoulé cattle. A previous GWAS for trypanosome infection status and level of parasitaemia for groups of artificially infected African taurine, Zebu and their crosses was performed by Hanotte *et al.* (2003). The signals on CHR16 in Hanotte *et al.* (2003) overlap with the signal found in the current study. We also compared our GWAS signals with a range of studies on selection signatures in African cattle (Dayo *et al.*, 2009; Gautier *et al.*, 2009; Smetko *et al.*, 2015; Tijjani, 2019), for which trypanosomis was considered a very strong driver of past selection. Only one common result was observed



Source: Authors' own elaboration.

Figure 1: Manhattan plot for the Trypanosomosis status in the Baoule cattle (The upper solid line is the Bonferroni threshold, the lower solid line an indicative threshold at -log10 *p*-value = 5.)

Table 1: SNPs with genome wide significant effects for trypanosomosis status

Chromosome	Name	Position (bp)	*P*-value
16	BTA-109095-no-rs	6 452 389	1.74e-08
22	BovineHD2200011407	39 725 145	3.95e-08
8	BovineHD0800017070	56 636 956	9.97e-07
9	BovineHD0900012349	44 431 079	1.01e-06
24	Hapmap38329-BTA-57629	22 454 666	3.91e-06
16	UA-IFASA-4552	56 715 642	5.17e-06

Source: Authors' own elaboration.

between those studies and ours. The second strongest signal in our study, within the protein tyrosine phosphatase receptor type G (PTPRG) on CHR22 (Mb 39.0-39.7), was considered an indicator of tropical adaptation in African Zebu cattle (Tijjani, 2019).

4. Conclusions

In the present study, SNP associations indicated regions potentially related to trypanosome prevalence in six regions of the genome in Baoulé cattle. The signals were not very strong, potentially because of the small sample size. Another plausible explanation for weak results is that the most important genes that provide the Baoulé with its typanotolerance (relative to susceptible breeds), may already be fixed at the favourable allele in the population. The protein tyrosine phosphatase receptor type G (PTPRG) on CHR 22 (Mb 39.0-39.7) is putative candidate trypanotolerant gene in our study. A GWAS for trypanosome infection status including Baoulé, Zebu and their crosses in the southwest of Burkina Faso is under way.

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Preparation and evaluation of dual protected nutrients for dairy cattle: An *in vitro* study

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Abstract

An *in vitro* trial was carried out to study the degradability of soybean meal following dual protection of protein and fat. The dual protection of nutrients was done by oil and aldehyde treatment. To begin, the crude protein (CP) content of soybean meal (SBM) was determined. One portion of SBM was then coated with rice bran oil at a 2:1 ratio and stored. The other portion was finely ground and treated with formaldehyde at 1 percent of its crude protein content and kept overnight. One part of oil-coated SBM was mixed with 2 parts of formaldehyde-treated SBM and mixed thoroughly. Four replicates of each mixture were subjected to two-stage *in vitro* degradability studies against a control for three times. The substrate was analysed for contents of dry matter (DM), organic matter (OM), crude fat (CF) and CP. The collected data were subjected to *t*-test statistical analysis. The *in vitro* CP and CF digestibility values of the protected mixture (36.23 percent vs 25.68 percent) were less than for the unprotected samples (52.82 percent vs 72.89 percent). The *in vitro* DM and OM degradability values were also significantly ($p < 0.05$) lower in the protected group than in the unprotected group. We concluded that oil and formaldehyde treatment together will reduce ruminal degradability of both protein and fats in SBM and rice bran oil.

Keywords

crude protein, dual protection, oil, degradability, dry matter, formaldehyde.

1. Introduction

Livestock agriculture is an important allied sector for livelihood income generation for farmers in developing countries. Within the livestock sector, ruminant animals - mainly cattle, buffaloes, sheep and goats - occupy a major role because they mainly survive on the utilization of the forage-based feeding. They are effective bio-converters of poor-quality forage into valuable goods, without competing with foods for human consumption. For this bioconversion, the microbes present in the rumen of the animals play an important role. The rumen microbes degrade dietary protein sources, regardless of their quality, into ammonia and amino acids and then incorporates these nitrogen sources into microbial proteins. Ruminants derive their amino acids jointly from dietary protein (escaping rumen degradation) and as well as from microbial protein synthesis (from degraded proteins) in the rumen. The amount of protein and amino acids that escapes rumen degradation varies greatly among feeds, depending on their solubility in the rumen and the rate of passage into the small intestine. In the case of high producing animals, the microbial protein alone is usually not sufficient to meet their protein requirement. In such cases, feeding of animals with protected proteins (bypass protein) is needed to meet their requirements (Kumar *et al.,* 2016). By following the protection technology, the nutrient requirements for high milk producing animals can be met (Parnerkar *et al.,* 2010). Ferguson, Hemsley and Reis (1967) suggested that the most effective means of supplementing protein to ruminant animals is protecting them from rumen degradation. The protection could be achieved by feeding less degradable

protein sources (like fish or blood meal), or by applying heat or chemical treatment. The protection of casein from rumen degradation has been demonstrated by treatment with formaldehyde. This protected nutrient technology is not limited to only lactating animals; when fed to young calves, an increase in the average daily gain in body weight has been observed (Patel, Gupta and Jani, 2020). Further, the digestibility of this casein in the lower tract has been indicated by increases in wool production, weight gain, nitrogen retention (Tandon and Siddique, 2016) and post ruminal non-ammonia nitrogen concentration (Wadhwa and Bakshi, 2004).

Jadhav *et al.* (2018) demonstrated that ammonia production was totally inhibited by 0.5 percent and 1.0 percent formaldehyde treatment of oil cakes. The author further suggested that microbial dry matter degradability decreased significantly with each increment of formaldehyde. Enzymatic and total digestion were also affected by formaldehyde treatment. Further, *in vitro* digestion of soybean meal dry matter, organic matter and nitrogen can be reduced by the addition of formaldehyde. This reduction may be due to the lack of free nitrogen for microbial growth or to the protection of other dietary components besides protein by the addition of formaldehyde. Various forms of aldehydes have been used at different concentrations for protection of degradable proteins. Khakhil *et al.* (2015) suggested that the maximum depression of *in vitro* ammonia production could be achieved by using 0.6 percent formaldehyde or 1.5 percent glutaraldehyde or glyoxal. Treating high quality protein or amino acids or both with aldehydes to decrease their degradation may have a practical value in feeding ruminant animals.

Formaldehyde has been widely used in animal feeds. For example, in monogastric animal feeds (poultry and pigs), formaldehyde is often sprayed over feeds to reduce the microbial contamination as well as to increase the shelf life of the feed. In ruminant diets, formaldehyde is especially used in production of protected proteins. The major advantages of the formaldehyde treatment method when compared to the other treatments are the following: i) most of the formaldehyde treatment processes are carried out in a sealed container and this results in complex HCHO binding and methylene bridges that resist microbial degradation in the rumen; ii) the extent of protection is adjustable based on the feed ingredients, thereby reducing instances of over- or under-protection; iii) it provides the maximum availability of essential amino acids; iv) it does not alter the acid- and neutral-detergent insoluble nitrogen levels and also reduces the pathogenic (e.g. Salmonella and mould) microbe populations; and v) formaldehyde is cheaper than heat treatment. Due to these advantages, formaldehyde method of protection was preferred over other methods.

Similar to protein, the fats added to ruminant diets may also need to be protected, as they can affect fibre digestibility if fed at high levels. Generally, fats are fed as calcium salts of fatty acids or encapsulated. Various methods are often used separately for the protection of each of these nutrients (fats and proteins), which further adds to the cost of production. Hence, the present study was carried out to develop a technique to protect both the fat and the protein from rumen degradation in a single procedure. The developed dual protected product was further evaluated for the *in vitro* degradability of the protein and fat.

2. Materials and methods

Materials

Soybean meal (Figure 1) and crude rice bran oil (Figure 2) used for cattle feeding were purchased from the local feed supply market in Namakkal district, Tamil Nadu Province, India. The formaldehyde (Figure 3) used for the study was obtained from Sigma-Aldrich.

Oil coating of soybean meal

The coating of the protein sources with lipid substances (Sklan and Tinskey, 1993) is a protective method against microbial degradation in the rumen. The coating of protein with lipid substances decreases the rumen degradability. The effect of protection generally increases with increasing amounts of the coating agent. In this study,

Source: Authors' own elaboration.

Figure 1: Soybean meal

Source: Authors' own elaboration.

Figure 2: Rice bran oil

Source: Authors' own elaboration.

Figure 3: Formaldehyde

a 2:1 ratio (i.e. 2 parts soybean meal, 1 part oil) was used for the coating of soybean meal. For the rice bran oil, 100 ml was mixed with 200 g of finely ground soybean meal. The mixture was kept aside.

Formaldehyde treatment of soya

Usually, 0.5-1.5 percent of crude protein of the concentrate feed is used for the preparation of protected protein. In the present study, 1.0 percent of formaldehyde was used for the preparation of the bypass protein. Commercial-grade Formalin (40 percent Formaldehyde) was available (40 ml formaldehyde = 100 ml water). For the soybean meal had 45 percent crude protein, 1 percent of this fraction corresponded to 2.25 ml of formalin for this treatment. The formalin was sprayed over 200 g of finely ground soybean meal and mixed properly. The treated soybean meal was then kept overnight to remove the odour.

Preparation of soybean meal treated with oil coating of formaldehyde

One part of the oil coated soybean meal was mixed with two parts of formaldehyde treated soybean meal to yield the mixture that was used for the *in vitro* degradability trail.

In vitro degradability study

The *in vitro* degradability study was conducted with the prepared sample by applying the Tilly and Terry method (1963). Rumen liquor was obtained from a dairy cow by using a stomach tube and the contents were screened through muslin cloth to remove the particulate materials. The rumen inoculum was then combined with micro-nutrients in four fermentation flasks (Figure 4) per sample that were then flushed with carbon-dioxide to maintain anaerobic conditions. Subsequently, the flasks were sealed and incubated at 39 °C for 48 hours. Similarly, a parallel set of control flasks containing un-treated soybean meal without fat coating was also maintained using same inoculum. Following this 48-hour period, the medium in each flask was passed through filter paper and the dry matter, crude protein, and crude fibre contents were determined as per AOAC (2016). The obtained data were subjected to the paired *t*-test to determine the significance of differences in treatment means.

Source: Authors' own elaboration.

Figure 4: *In vitro* fermentation flask

3. Results and discussion

The *in vitro* crude protein and crude fat digestibility in the protected mixture (36.23 percent vs 25.68 percent) were lower than the unprotected (52.82 percent vs 72.89 percent). The *in vitro* dry matter degradability was significantly ($p < 0.05$) lower in the protected group than in the unprotected control (Table 1).

The undegraded protein of untreated soybean meal was 17.52 percent (Table 1). In the meal treated with oil coating and formaldehyde, the mean undegraded portion was 38.17 percent. Therefore, oil coating and formaldehyde treatment is effective in protecting the material from the rumen microbes. The dry matter availability to the animal is therefore presumably improved.

Table 1: Effect of dual protection of nutrients on the *in vitro* degradability.

Parameter	C1	C2	C3	C4	T1	T2	T3	T4
DM% of the sample after treatment	0.108	0.110	0.103	0.055	0.154	0.150	0.266	0.234
Protein degradability (%)	80.244	79.717	80.559	89.361	72.261	70.406	51.553	53.079
Amount rumen un-degraded (%)	19.736	20.283	19.441	10.639	27.738	29.594	48.447	46.921

Average undegraded portion of control soybean meal = 17.52 ± 2.30

Average undegraded portion of dual treated soybean meal = 38.17 ± 5.51

p-value of difference in means: 0.001

Source: Authors' own elaboration.

Note: C1-C4: Untreated soybean meal (control samples); T1-T4: Dual protected soybean meal (treated samples).

These results agreed with the findings of Ferguson, Hemsley and Reis (1967) and Peter *et al.* (1971), who reported a reduction in rumen degradability of treated protein sources and more amino acid availability in the small intestinal level. This additional availability of the amino acids and fatty acids could provide more building units for milk protein synthesis, resulting in greater milk yield and fat content. In short, the dual protection of

nutrients will theoretically provide higher nutrients at the absorption site for better utilization, which could result in improvements in both the quality and quantity of milk produced, especially in early lactation when demand for protein and energy are high.

4. Conclusions

Dual treatment of oil and formaldehyde together helped to reduce the *in vitro* ruminal degradability of both protein and fats. Application of this treatment might be especially useful during the early lactation period, without the need for separate sources of rumen-protected protein and fat. However, this experiment was done *in vitro*, so *in vivo* studies would be needed to confirm this hypothesis.

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Application of improved technologies for sustainable livestock productivity: The way forward

Genomic technology contributing to sustainable livestock development in China

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Today we cannot emphasize enough the importance and impact of sustainable livestock in achieving agriculture and food security in the world. As stated by FAO and OECD in 2019 (OECD and FAO, 2019), for instance, the consumption of meat, milk and eggs in large parts of Asia and Africa is expected to grow between 200 - 600 percent in the next 30 years. Livestock contribute 40 percent of the global agricultural output and support the livelihoods and food security of almost a billion people.

In Asia and Africa, livestock are exposed to a range of high impact diseases, such as tuberculosis, brucellosis, chronic parasitosis and numerous vector-borne infections. Multiple infections with these diseases cause death and production losses, estimated to be as high as 40 percent.

According to "The China Agricultural Outlook Report (2021-2030)" (CAS, 2021), in the next ten years the total consumption of meat, poultry eggs, dairy products and aquatic products in China is expected to have a significant growth.

One of the implications of such growth is on the increased importance of feed, in terms of both quantity and quality. In China food security is largely a matter of livestock feed security. Rapid growth of the livestock sector will lead to continued increases in feed consumption demand. The total consumption of corn and soybeans, for instance, are expected to grow at an average annual rate of 1.1 percent and 0.9 percent, respectively. Table 1 shows the ten-year (2020-2030) trend of pork production, consumption and importation in China, while Figure 1 shows the trend of livestock production and consumption in China, based in the China agriculture outlook report (2021-2030) (CAS, 2021).

As indicated in Figure 1, pork production is expected to increase by almost 20 million tonnes by 2030 compared

Table 1: Ten-year trend of pork production, import and consumption in China* (million tonnes)

	2020	2021	2025	2030
Pork production	41.13	49.27	57.72	59.98
Pork import	4.39	3.80	1.30	1.20
Pork consumption	45.45	52.97	58.90	60.98

Source: CAS. 2021. *The China Agricultural Outlook Report (2021-2030).* Beijing, Chinese Academy of Agricultural Sciences. http://english.moa.gov.cn/news_522/202104/t20210428_300641.html

Note: *Data from The China Agricultural Outlook Report (2021-2030)

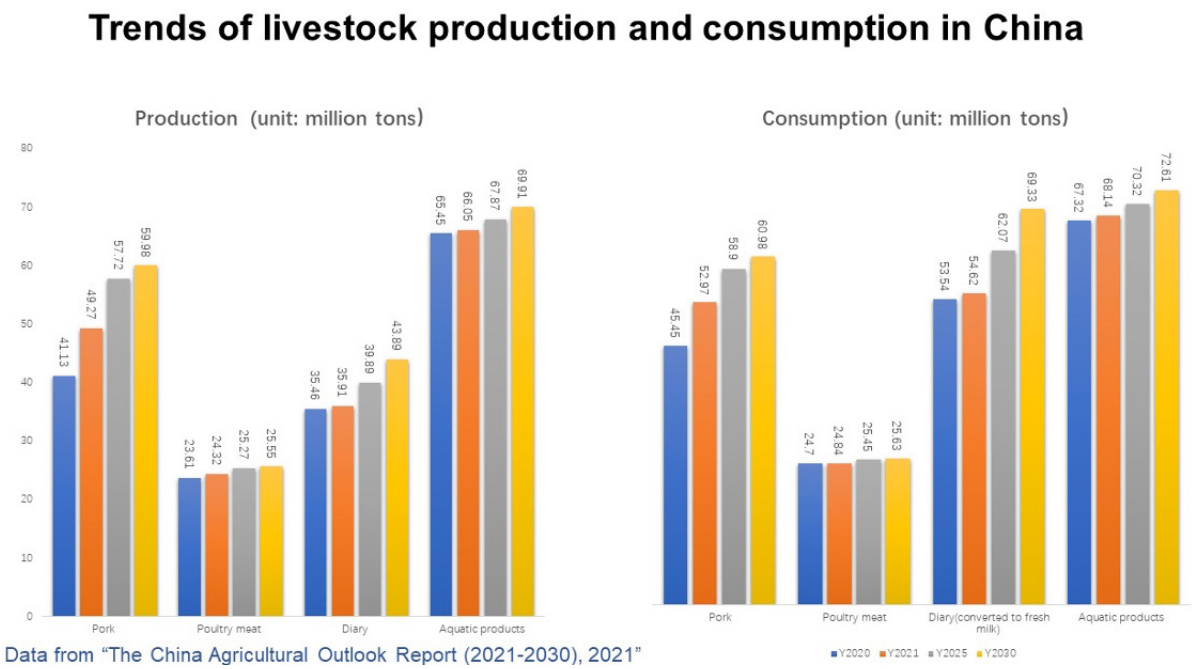

Figure 1: Trends of livestock production and consumption in China

with the production of 2020. The production of poultry meat will not increase as dramatically as the production of pork. Dairy production is expected to increase by nearly 9 million tonnes between the years 2020 and 2030. Production of aquatic products was already quite high in 2020, and is predicted to increase by about 5 million tonnes by 2030.

Looking at consumption (Figure 1), we can see that poultry meat consumption again will have a nearly balanced consumption in the year 2030, with only a small increase expected compared with that of 2020. Dairy and aquatic product consumption are forecast to show significant increases by 2030, leaving a significant gap between predicted production and consumption.

In the meantime, new technologies present new opportunities to enhance sustainable livestock development. Taking advantage of the power of modern genomic and phenomic technologies, as well as big data, there is a big push to accelerate precision plant and livestock breeding. In the livestock sector, the progress made in the improvement of livestock genetic resources will particularly be a strong catalyst. It has been well known that the value of the germplasm resources is their DNA information as much as the material itself. Therefore, developing a "digital genebank" of DNA data and associated phenotypic data will transform the traditional genebanks, as well as livestock breeding in general.

The increased development and utilization of such applications of genomic technologies has been largely driven by the rapid development in high throughput and low-cost genome sequencing during the past 20 years. We know that the completion of first human genome around the end of the last century was the result of 13 years effort of scientists from six countries at the cost of over 3 billion US Dollars. Today the sequencing of a whole human genome costs less than USD1 000 and is done in a matter of hours.

The Nature Methods magazine named genome editing as the method of the year in 2011 (Nature Methods, 2012). In 2015, the discovery and development of CRISPR/Cas9 tools was named by the Science magazine as the breakthrough of the year (Travis, 2015). Last year the Nobel Prize in Chemistry was awarded to two distinguished scientists and key players in the development of genome editing.

In 2019, Science magazine published a special report about the development of CRISPR technologies in China (Cohen, 2019). The report specifically cited some leading scientists, for instance, Dr Caixia Gao, Dr Jiayang Li and Dr Jiankang Zhu of the Chinese Academy of Sciences. That report also shared some interesting figures, such as that the number of publicly available CRISPR patent applications in China was just slightly less than that of United States while far exceeding the rest of the world. In terms of published papers in the application of CRISPR, Chinese scientists had published about 2 000 papers by the end of 2018, while American scientists had published about 3 000 papers during the same time.

The BGI Institute of Agricultural Research has carried out some research on the application of genomic selection for the breeding of livestock species, for instance for cashmere goat (Li *et al.*, 2017). The target trait was the cashmere fineness, and a breeding nucleus of the cashmere goat was constructed using genomic selection methods. Several candidate genes associated with cashmere diameter and curliness identified through the genome-wide association study (BGI Inst. Ag. Res., unpublished data 2020).

Genomic analyses can be an effective tool to enhance local farm animal genetic resources. The research at the BGI Institute of Agricultural Research successfully rebuilt the pedigree of a local breed of pig from Hunan Province, as well as a Guangxi duck breed in Southwest China. The genome-based population genetic analysis helped to optimize the breeding plans. (BGI Ins. Ag. Res. Unpublished data 2020)

The China National GeneBank (CNGB) at Shenzhen is a modern platform for genomic sciences. Established in 2016, CNGB is committed to supporting public welfare, life science research and industry incubation, through effective bioresource conservation, digitalization and sustainable utilization.

A major feature of CNGB is its integration of storage, "reading" (genomic digitalization) and "writing" (genome editing and DNA synthesis). It is a genebank which goes much beyond cold storage of animal germplasm and crop seeds.

The genebank has a high capacity for storing bio-samples, and serves as a biorepository of plants, animals, microorganisms as well as human samples. It is also a repository or a data centre for these biological resources.

CNGB has perhaps the largest capacity in the world in genome sequencing, which is about 24 petabits of data per year. That corresponds to a capacity of about 240 000 whole human genome sequences. That system is fully automated and records all information in real time.

Single cell sequencing is a rapidly developing new technology. It provides genomic information at the individual cell level. This exciting and cutting-edge method will help answer fundamental questions in many aspects of biology as well as serve as a guide to unravel the secrets of human diseases. For example, the human disease cell atlas developed by single cell sequencing will enhance our understanding, diagnosis and treatment of many diseases, including cancer.

The CNGB has built an automated single cell sequencing platform, which covers sample preparation, sequencing preparation, high throughput data analysis and sharing. The so called ATAC-seq stands for "assay for transposase-accessible chromatin using sequencing", a technique used in molecular biology to assess genome-wide chromatin accessibility.

The rapid development and application of genomics, trans-omics and big data are transforming agricultural research and development and biodiversity conservation. Systems research, enhanced with new tools, is driving the development of new value chains, new products and production systems, as well as the new continuum of agriculture – food – nutrition – health.

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Application of nuclear and genomic technologies for improving livestock productivity in developing world: Challenges and opportunities

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Abstract

The huge benefits of genomic technologies have been well demonstrated in developed countries. This has been facilitated by the existence of well-established systems for data recording, genetic evaluation and delivery of superior genetics. Moreover, impetus has been provided by economic drivers to support public and private investment in research and genotyping. In addition, effective organization has been key to this huge success in terms of the formation of strategic cross-country collaborations for genotyping of genetically connected herds and key individuals. Some potential "quick wins" of genomics in developing countries include the ability to handle the limited data structure in these countries that has resulted from lack of pedigrees and small herd and sire progeny sizes. These quick wins could be achieved through the use of the genomic relationship and single step genomic best linear unbiased prediction methodology for the prediction of genetic merit and the development of tools for determining parentage and breed composition. The accuracies of genomic predictions reported for these developing country systems vary from low to high, but nevertheless provide an opportunity to select top ranking animals. Indigenous breeds represent a unique set of genotypes adapted to surviving under harsh conditions. Genomics provide a means of understanding and utilizing the underlying genetics and this will become even more important in the light of climate change. Genomic approaches aimed at enhancing feed production by increasing the yield and quality of grain and forage crops can provide indirect benefits in terms of increasing animal productivity. However, the cost efficiency of genomic technologies remains one of the limiting factors for their widespread usage in developing countries. There is the need to establish a one stop shop to offer bundled services such as genotyping, parentage and breed composition, genomic selection and mating advice to increase the cost efficiency. The joint application of genomic and reproductive technologies has been found to improve cost efficiency. Finally, use of digital tools to build efficient and routine data collection systems will be critical. To increase efficiency and maximize impact, genomic systems must be linked to existing systems such the national artificial insemination centres, for the delivery of superior genetics to the farmer.

Keywords

genomic technologies, developing countries, livestock, indigenous breeds, genotyping

1. Introduction

The benefits of genomic selection have been well demonstrated in developed countries and these include higher rates of genetic gain, especially in dairy cattle, due to reduced generation intervals and high accuracies for young genomic proven bulls of above 70 percent for production traits. In the United States of America, for instance, young genomic sires accounted for 67 percent of artificial insemination (AI) breeding in 2019 (CDCB, 2019). In addition, high accuracies for traits of low heritability have been reported for cows and genomic

selection (GS) has also enabled genetic improvement in difficult to measure traits such as feed intake and methane emissions, which are of global importance with respect to climate change (Pryce and Haile-Mariam, 2020). The tremendous genetic gains from the application of genomic technologies in developed countries could be attributed to various factors including the existence of well-established systems for data recording, genetic evaluation and delivery of superior genetics; and the impetus provided by major drivers such as governments, multi-national breeding companies or breed societies through financial investment to support necessary research and genotyping costs of bulls. In addition, effective organization and design have also been key to this huge success in terms of the formation of across-country collaborations such as the consortium among several European countries (Lund *et al*, 2011) or among North American countries, the United Kingdom of Great Britain and Northern Ireland and Italy aimed at increasing the size of their reference populations, and the formation of systems that enabled joint genomic predictions. Specific examples include Inter-genomics for the Brown Swiss Breed (Hossein *et al*, 2011) and the strategic genotyping of connected herds to handle difficult to measure traits as in the Australian AGIN project (Pryce *et al.*, 2017). Lastly, farmers have also played an important role, as the genomic systems have been designed to address farmer's needs; helping them to select which calves to keep or cows to flush or breed with sexed semen and pedigree validation. Consequently, the United States of America evaluations in September 2021, for instance, included more than one million genotyped cows, which are mostly from individual farmers (CDCB, 2021).

Therefore, challenges and opportunities of nuclear and genomic technologies in developing countries should be examined not only in terms of the direct application of the principles of genomic selection per say, but also, the important associated factors such as infrastructure, design, organization and the roles of farmers.

2. Some opportunities of genomic technologies

Overcoming limited data structures

Initially, we will examine the potential quick wins of genomic technologies, given the population and data structure of livestock in developing countries. The lack of proper data and pedigree recording schemes has been one of the major bottlenecks to genetic evaluation and the implementation of breeding programmes in developing countries (Kosgey and Okeyo, 2007). However, when performance data and genotypes are available, the latter implies that the genomic relationship matrix can be computed, thereby enabling the prediction of the genetic merit of animals, with less reliance on pedigree information. Brown *et al.* (2016) presented accuracies of genomic predictions for smallholder dairy daily milk yield in Kenya in a data set with no pedigree information. Similarly, for crossbred dairy cattle in India, Al Kalaldeh *et al.* (2021) undertook genomic evaluation for daily milk yield using only genotypic data. Mrode *et al.* (2021) reported genomic prediction for daily milk yield and body weight for crossbred cattle in the United Republic of Tanzania using genomic relationships. In fact, of the 1906 cows involved in the study, the available pedigree indicated that 88.14 percent of these cows had no recorded parent, while only 0.63 percent and 11.23 percent, had both parents, and one parent identified, respectively. This underlines the importance of the availability of genotypic information in enabling prediction of the genetic merit in smallholder systems, as the pedigree relationships were clearly inadequate.

In addition to the lack of pedigree data, the small herd sizes in developing countries imply that animal and herd effects are often completely confounded. In the genomic prediction of dairy cattle reported for milk yield and body weight for the United Republic of Tanzania by Mrode *et al.* (2021), more than 50 percent of the cows were the only animal in their respective herds. However, through the use of the genomic relationship matrix, haploblocks from common sires used across herds enabled the separation of herd and animal effects (Powell *et al.*, 2021).

In general, the use of single step genomic best linear unbiased prediction ssGBLUP methodology (Mizstal *et al*, 2009) has made genetic prediction more feasible in developing countries, as this reduces the reliance on having a sufficient number of males with prior estimates of breeding values of reasonable accuracy. The use of ssGBLUP implies that genotyped and ungenotyped animals can be jointly analysed, resulting in an increased accuracy of predictions. Also, strategic genotyping and phenotyping can be used to handle difficult to measure traits where only a proportion of the animals have been genotyped.

Given the limited data sizes in developing countries, across country genetic evaluation is appealing, as it has the advantage of increasing the accuracy of evaluations and making superior bulls available for use across countries (Opoola *et al.*, 2020). However, such joint evaluations suffer from a lack of adequate genetic connectedness when good pedigree information is not available (Opoola *et al.*, 2020). Thus, genomic approaches that involve pooling genotypes and data across countries for joint genomic evaluations, such as in the case for the Brown Swiss cattle (Hossein *et al*, 2011), increase the prospects of across country evaluations. The effectiveness of such a joint evaluation is yet to be demonstrated in crossbred cattle of different breed compositions reared across countries, however, which is often the case in developing countries.

Thus, genomic approaches have made it possible to undertake the prediction of genetic merits in developing countries with limited pedigree and data structure. The accuracies of genomic predictions from these studies have ranged from low to high values, depending on the country and trait. The genomic accuracies reported by Mrode *et al.* (2021) for milk yield and body weight varied from 0.55 to 0.83. Genomic prediction accuracies reported for carcass traits (rib eye area (REA), back fat thickness (BT), and hot carcass weight (HCW)) in Brazilian Nellore cattle (Fernandes Júnior *et al.*, 2016) were low to moderate 0.21 (BT), 0.37 (HCW) and 0.46 (REA). Silva *et al.* (2016) investigated genomic selection in an experimental farm with 788 Nellore animals genotyped with high density (HD) single nucleotide polymorphism (SNP) array, and included a pedigree of 9551 animals and reported accuracies of 0.30 for feed conversion ratio and 0.45 for residual feed intake from a ssGBLUP analysis. A summary of accuracies from genomic studies in developing countries has been presented by Mrode *et al.* (2019), but more recently Al Kalaldeh *et al.* (2021) reported an accuracy of 0.42 for smallholder crossbred cattle in India for adjusted test day milk yields. The estimates of accuracy for several methods for test day milk yields and body weight in the United Republic of Tanzania (Mrode *et al.*, 2021) are reported in Table 1.

Table 1: Forward validation results for daily milk yield (kg) and body weight (kg)

Trait	Method	Correlation	Regression
Milk yield	FRM-1	0.57	1.1
	FRM-2	0.59	1.0
	RRM-1	0.55	1.0
	RRM-2	0.53	0.92
Body weight	FRM-1	0.83	1.0
	FRM-2	0.77	1.1

Source: Mrode, R., Ojango, J., Ekine-Dzivenu, C., Aliloo, H., Gibson, J. & Okeyo, M.A. 2021. Genomic prediction of crossbred dairy cattle in Tanzania: A route to productivity gains in smallholder dairy systems. *Journal of Dairy Science*, 104(11): 11779–11789. https://doi.org/10.3168/jds.2020-20052

Note: FRM-1: GBLUP fixed regression model; FRM-2: ssGBLUP fixed regression models. RRM-1: GBLUP random regression model; RRM-2: ssGBLUP random regression model.

3. Parentage and breed composition determination

In developing countries and especially for dairy cattle, crossbred animals make a huge contribution to the amount of milk produced in the dairy sector. Usually, the parentage and breed composition are unknown due to a lack of adequate pedigree recording. An accurate estimation of the breed composition in smallholder systems is very important in determining the breed composition most suitable for the given agro-ecological zones. The application of SNP information for the determination of breed composition and parents represents one of the quick wins of genomics in developing countries. Working with crossbred dairy cattle in eastern Africa, Strucken *et al.* (2017) developed an assay of 200 SNPs for the determination of the breed composition of animals. However, the number of SNPs increased to 400, if parentage verification was to be incorporated. Al Kalaldeh *et al.* (2021) estimated breed proportions using SNP data in genomic prediction for a smallholder crossbred dairy production system, and also in determining the performance of these crossbred animals under different environments

Understanding the genetic basis of adaptation in indigenous breeds

Indigenous breeds represent a unique set of genotypes adapted to harsh conditions and are often resistant to endemic diseases and pests. In the light of climate change, understanding the genetic basis for these adaptive characteristics will be very important in incorporating relevant genomic regions in breeding programmes to mitigate the impact of climate change on animal productivity. Genomics provide a means for understanding, and therefore, utilizing the genetic information that underlies this adaptation in indigenous livestock. In a research study involving 12 indigenous African cattle breeds, Kim *et al.* (2020) identified several loci in African cattle related to general immunity, heat-tolerance, trypanotolerance and reproduction. Similar research in small ruminants has identified genomic regions related to adaptation to arid environments and high altitude and resistance to endoparasites in sheep from Tunisia and Ethiopia (Ahbara *et al.*, 2021; Wiener *et al.*, 2021). Some of the results from these studies could be utilized in breeding programmes, including the incorporation of functional regions/genes in genomic prediction for increased accuracy, using Bayesian methods such as BayesR (Erbe *et al.*, 2012). However, if the genomic regions were identified precisely, methods such as gene editing could be used to incorporate these regions into breeding animals, including breeding males to be used as surrogate sires.

Indirect effects thorough improving animal feeding resources

Feeds account for about 70 percent of the production cost of most livestock species. Feed nutritional quality can be highly variable, and it is usually expensive and difficult to

measure. Therefore, genomic approaches that will enhance feed production and quality provide an indirect means of increasing animal productivity. One of the interesting approaches in this area is the application of genomics to improve straw or stover quality of dual-purpose crops for utilization by animals. Vinayan *et al.* (2021) presented validation results from a genomic prediction analysis to improve maize stover traits such as *in vitro* organic matter digestibility (IVOMD) and metabolizable energy (ME). The aim of the study was to investigate the development of new dual-purpose maize varieties with grain yield optimized while the nutritional quality of the stover is improved for animal feed. Using a SNP panel of about 3 000, they reported validation accuracies of about 0.45 for the prediction of IVOMD and ME in maize seedlings, based on using genotypic selection only; indicating the feasibility of selecting stover quality for animal feed at an early stage. GWAS studies in the perennial forage Napier grass, aimed at implementing marker assisted selection, have also identified several significant marker associations for agronomic, morphological and water-use efficiency traits (Muktar *et al.*, 2022).

4. Some challenges for widespread usage of genomic technologies

Inadequate systems for data collection

While genomics has provided a pathway to overcome some of the limited data structures for livestock in developing countries, the lack of systems for routine data recording still constitutes a major obstacle for its widespread usage. As United Kingdom of Great Britain and Northern Ireland animal scientist Mike Coffey (Pérez-Enciso and Steibel, 2021), rightly remarked, "In the age of the genotype [genomics], phenotype is king." This is due to the fact that one of the major determinants of the efficiency of genomic selection is the size of the reference population, that is, animals with both genotypic and phenotypic data required for the estimation of the SNP effects.

However, advances in information and communications technology (ICT) have enabled several development projects to pioneer the use of digital tools for performance recording in smallholder systems. Some of the projects applying modern technologies to capture performance data include the African Dairy Genetic Gains project (ADGG; www.ilri.org/research/projects/african-dairy-genetic-gains) operating in the United Republic of Tanzania, Ethiopia and Kenya, and sponsored by the Bill and Melinda Gates Foundation. The ADGG has used a software based on the Open Data Kit, installed in tablets and mobile phones of staff employed as performance recording agents. The performance records captured include daily milk yield, heart girth for the prediction of body weight, body condition score and AI data for dairy cattle. Similarly, the collection of performance data on a large scale by ICT in India by the Bharatiya Agro Industries Foundation (BAIF) was reported by Ducrocq *et al.* (2018), which involved about 170 AI technicians equipped with multi-component software, installed first on dedicated "data loggers" and later on mobile phones.

In small ruminants, data collection has mostly been implemented through community-based breeding programmes (CBBP) in Ethiopia and Malawi for growth traits, twinning rate and fleece weight (Haile *et al.*, 2019). To overcome some of the challenges of internet connectedness, a digital system, AniCloud (https://anicloud.com), integrated with AniCapture, a smart device software designed for offline data gathering, has been employed. A summary of some of the initiatives on the application of ICT and mobiles phones and other digital tools for data collection in developing countries (Mrode *et al.*, 2020) is presented in Table 2.

The recording of fitness related traits such as fertility and disease poses a greater challenge in smallholder systems compared to measuring growth and production related traits. However, some emerging innovative approaches to capture fertility data, including oestrus detection using pedometers to measure a cow's activity, have been investigated in smallholder dairy farms (Muasa, 2019). Generally, cows in heat are restless, resulting in increased movement (Baxter, King and Hurnik, 1977) and the activities of the cows such as the number of steps made per unit time can be captured through sensors attached to the hind leg. The measurements of the cow's activities can then be used to predict progesterone profile. Muasa (2019) investigated the sensitivity and specificity of three different oestrus detection technologies (P4 rapid, CowAlert® and Estrotect™) on a large-scale farm in the United Kingdom of Great Britain and Northern Ireland and in smallholder dairy farms in Kenya and showed very comparable results across the farms of different scales. This indicates the feasibility of using some of these emerging tools to capture fertility in smallholder dairy systems.

When data capture is a major challenge as in smallholder systems, it is worthwhile investigating the minimum amount of data required to achieve predictions of the genetic merits of animals with good levels of accuracy, so that data collection systems can be simplified. Using the ADGG daily milk yield and genotypes, genomic prediction was investigated using GBLUP with partial lactation results compared with predictions based on 500 days in milk. The genetic parameters for milk yield and accuracy of genomic predictions are shown in Table 3. In general, heritability decreased as longer lactation lengths were considered. This finding is consistent with estimates of heritability for daily milk yield from a random regression model, with estimates varying from 0.33 on day 44 to 0.16 on day 404 (Mrode *et al.*, 2021). The rank correlations for all bulls

Table 2: Summary of digital tools that have been employed for performance data capture in some developing countries

Initiatives	Livestock	Tools	Traits recorded	Countries
African Dairy Genetic Gains Project	Dairy cattle	Mobile phones and tablets Open data kit and information and technology Platform called iCow (www.icow.co.ke)	Milk yield, body condition score, heart girth and insemination details	Ethiopia, United Republic of Tanzania, Kenya
Dairy Project Bharatiya Agro Industries Foundation (BAIF), India	Dairy cattle	Data loggers and mobile phones	Milk yield	India
Community Based Breeding Programme	Sheep and goats	Digital system, aniCloud (https://anicloud.com), and software aniCapture	Birth weight, body weight at various ages and twinning rate	Ethiopia, Malawi
Muasa (2019)	Dairy cattle	Pedometers	Cow activities for prediction of progesterone profile (fertility)	Kenya

Source: Mrode, R., Ekine Dzivenu, C., Marshall, K., Chagunda, M.G.G., Muasa, B.S., Ojango, J. & Okeyo, A.M. 2020. Phenomics and its potential impact on livestock development in low-income countries: innovative applications of emerging related digital technology. *Animal Frontiers*, 10(2): 6–11. https://doi.org/10.1093/af/vfaa002

Table 3: Genetic parameters and accuracy of predictions using part-lactation data

	Days in milk				
	100	200	300	400	500
N	4 400	8 886	13 177	17 005	19 599
Heritability	0.19 ± 0.05	0.17 ± 0.04	0.16 ± 0.04	0.14 ± 0.03	0.11 ± 0.03
	Rank correlations of genomic estimated breeding values with those from 500 days in milk				
All bulls	0.87	0.93	0.97	0.99	
Top 20% of bulls	0.30	0.75	0.75	0.79	
	Genetic prediction of 276 young animals born after 2014 with records excluded				
Accuracy	0.44	0.52	0.54	0.57	0.58
Regression	0.83	0.95	0.97	1.04	1.06

Source: Authors' own elaboration.

from evaluations based on 300 days in milk (DIM) and 400 DIM were 0.97 and 0.99 compared with those from 500 DIM, indicating little change in ranking using these three categories of DIM. However, when only the top 20 percent of bulls were considered, these rank correlations were much lower at 0.75 and 0.79 for 300 DIM and 400 DIM, respectively, indicating there might be a loss of response if breeding decisions were based on only the top 20 percent of bulls using reduced data. Usually, the level of such re-ranking can be reduced by considering only bulls with high reliability, for instance, at least 80 percent. In terms of the accuracy and calibration of genomic predictions, the predictions from using 300 DIM and 400 DIM were very close to those based on 500 DIM, indicating that data collection for to up about 400 DIM might be adequate for genomic prediction. However, this study will further examine the number of actual milk test days recorded on each animal for a more meaningful conclusion.

Ascertainment bias

The opportunity that genomics offers to understand the genome diversity of indigenous breeds also poses the challenge of the inadequacy of available SNP arrays to unravel the genomic architecture of these breeds. Most commercial arrays are composed of SNP that have relatively high minor allele frequencies within the breed(s) from which the SNP were chosen (i.e. "ascertained"). These arrays thus ignore many highly variable SNP from other breeds. This process can lead to inaccuracies due to "ascertainment" bias, particularly the over-estimation of genetic variation within the breed(s) used to build the array. A recent study assessing the adequacy of 23 commercial bovine SNP arrays on two African cattle breeds (Ndama and Boran) and one European breed (Holstein), compared estimates of genomic parameters from the arrays to those based on whole genome sequencing (WGS). The numbers of WGS bi-allelic SNPs from Boran, Ndama and Holstein were about 33, 25 and 14 million, respectively, indicating greater genetic variation for the African breeds. Nevertheless, the commercial arrays showed greater estimated genetic variation for the Holstein. (Dr Abdulfatai Tijjani; personal communication). This raises the issue of ascertainment bias when using commercial SNP arrays, especially for GWAS or signatures of selection studies, to understand the genetic architecture of the indigenous

breeds. Thus, genotyping by sequencing (GBS) or low coverage sequencing approach may be a more cost-effective alternative strategy to study indigenous African cattle breeds.

Cost efficiency of genomic technologies

The cost efficiency of genomics constitutes another major challenge limiting the widespread usage of genomic technologies in developing countries. Currently, most genotyped animals in these countries are females and are mainly the outcome of development projects or genotyping activities by breed societies as in some cases in Brazil or South Africa. This is due to the lack of major economic drivers such as AI or breeding companies in these countries. In fact, most biological samples are sent abroad for genotyping due to the lack of adequate genotyping facilities. Therefore, the need for approaches to increase the cost efficiency of genomic technologies in developing countries is apparent. Such approaches may involve creating a one stop shop that offers marker genotyping services along with genomic tools for use of the information, such as for determination of parentage and breed composition, genomic selection and mating services. In addition, these shops could provide general data management and analyses, both to individual farmers and farmer organizations. In general, the cost efficiency of genomic technologies increases when combined with reproductive technologies such as sexing of semen for genomically proven young dairy bulls. Sexed semen would not only improve productivity but will almost ensure that the farmer will have a female calf for replacement purposes.

Studies have shown a similar trend for beef cattle, with more pronounced genetic gains realized if genomic selection is applied in combination with reproductive technologies. Carvalheiro (2014) examined the efficiency of genomic selection in the Nellore cattle breed under various assumptions. The approach that combined genomic selection with the use of *in vitro* fertilization for the production of embryos by a genotyped donor resulted in the greatest increase of genetic gain; about 79 percent higher compared to the basic breeding programme with half of the calves being born from AI proven bulls and the other half from natural mating sires.

To increase efficiency and maximize impact, genomic systems should be linked with existing systems for the delivery of superior genetics to the farmer. In some developing countries, national AI centres already exist for the promotion of AI and proven bulls to farmers. Genomic systems should be linked to such centres in those countries to ensure that semen of genomically proven bulls is delivered efficiently to farmers. However, in some countries, the use of AI is limited in scope and emphasizes imported semen. Thus, inclusion of locally sourced bulls to be used by farmers in the genomic evaluation system would require a significant paradigm shift.

5. Conclusions

Genomic technologies offer various opportunities to handle the limited data structure available in most developing countries in terms of using the genomic relationship matrix to undertake predictions when pedigrees are absent. In addition, when some pedigree data are available, genomic methods allow the joint analysis of genotyped and ungenotyped animals, thereby increasing the accuracy of predictions in these situations. Moreover, other quick wins for genomics in developing countries include parentage assignment and breed composition determination in crossbred dairy cattle, thereby facilitating the evaluation of appropriate breed composition for different production systems. The availability of genomic data offers the opportunity for across country or regional collaboration for increased accuracy and the use of the best sires across regions. However, widespread application will require establishing digital systems for more efficient routine data collection, increasing cost efficiency of genomic technologies through the offering of bundled genomic services, and strategic use of reproductive technologies.

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European Union actions for a sustainable and innovative animal nutrition sector

Author

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Abstract

The article introduces into the European Union's Farm to Fork Strategy, published in May 2020, and its approach as regards sustainable food systems. Focus is put on the primary production of food of animal origin. In the area of animal nutrition, the following actions are addressed in detail:

- More sustainable feed sources.
- Innovation in more sustainable feed additives.
- Modern feeding systems.
- Sustainable feed labelling for informed consumer choices.

1. Introduction – Farm to Fork Strategy

The Farm to Fork Strategy[1] (F2F) is at the heart of the European Green Deal[2] aiming to make food systems fair, healthy and environmentally-friendly. It follows a holistic approach, comprising the whole food chain, as shown in Figure 1.

Food systems cannot be resilient to crises such as the COVID-19 pandemic if they are not sustainable. We need to redesign our food systems which today account for a considerable share of global greenhouse gases (GHG) emissions, consume large amounts of natural resources, result in biodiversity loss and negative health impacts (due to both under- and over-nutrition) and do not allow fair economic returns and livelihoods for all actors, in particular, for primary producers.

Putting our food systems on a sustainable path also brings new opportunities for operators in the food value chain. The F2F aims to accelerate our transition to a **sustainable food system** that should:

- have a neutral or positive environmental impact;
- help to mitigate climate change and adapt to its impacts;
- reverse the loss of biodiversity;
- ensure food security, nutrition and public health, making sure that everyone has access to sufficient, safe, nutritious, sustainable food; and
- preserve affordability of food while generating fairer economic returns, fostering competitiveness of the European Union supply sector and promoting fair trade.

The European Union will **support the global transition** to sustainable agri-food systems through its trade policies and international cooperation instruments.

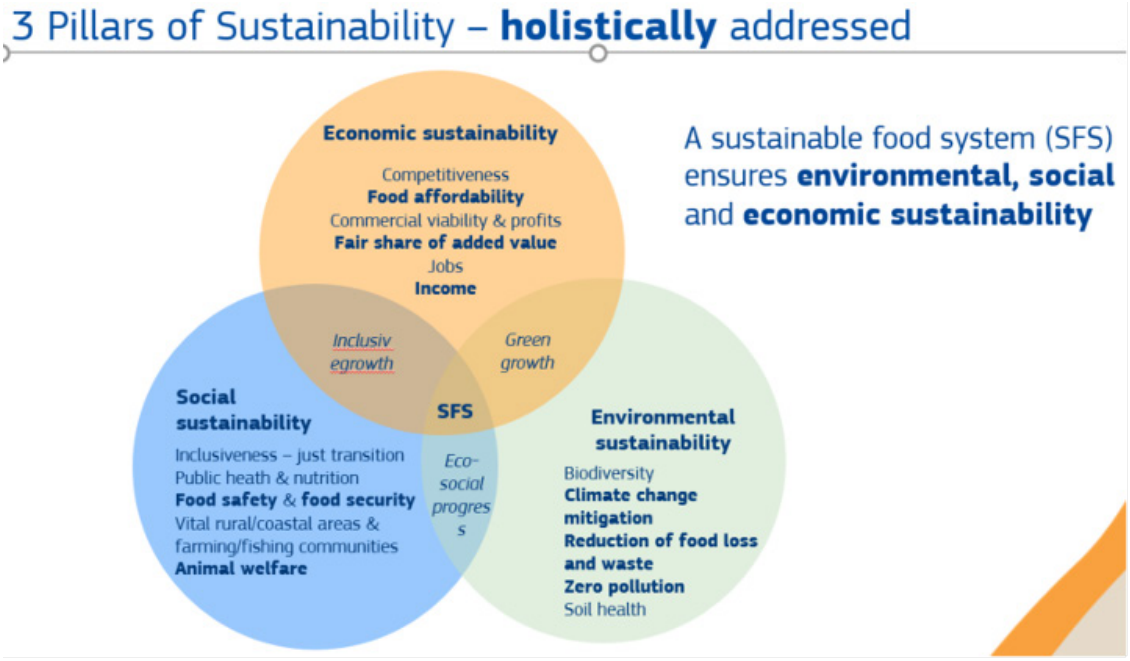
The concept of sustainability is based on three "Pillars": (i) economic stability; (ii) social sustainability; and (iii) environmental sustainability. These pillars and the interactions among them are illustrated in Figure 2.

Source: Authors' own elaboration.

Figure 1: Key elements of the European Union's Farm to Fork Strategy

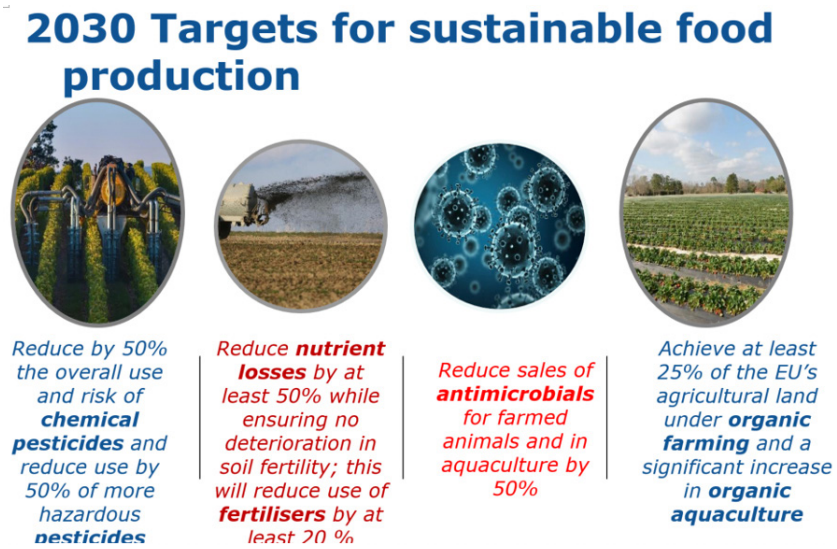
[1] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0381

[2] A European Green Deal | European Commission (europa.eu)



Source: Authors' own elaboration.

Figure 2: The key "Pillars of Sustainability" in the Farm to Fork Strategy



Source: Authors' own elaboration.

Figure 3: Targets of the Farm to Fork Strategy to be reached by 2030

The F2F has four targets, which are shown in Figure 3. Two of the four targets, on nutrient losses and antimicrobials, respectively, concern livestock farming.

In addition to the aspects addressed in the 2030 Targets, tackling food loss and waste is also considered key to achieving sustainability. Reducing food waste brings savings for consumers and operators, and the recovery and redistribution of surplus food that would otherwise be wasted has an important social dimension. It also ties in with policies on the recovery of nutrients and secondary raw materials, the production of feed, food safety, biodiversity, bioeconomy, waste management and renewable energy. The European Commission is committed to halving per capita food waste at retail and consumer levels by 2030 (Sustainable Development Goal Target 12.3). New methodology for measuring food waste will be applied to the data that Member States are expected to provide in 2022. This process will set a baseline and allow for the proposition of legally binding targets to reduce food waste across the European Union.

2. Role of animal nutrition in the development of sustainable food systems

Agriculture is responsible for 10.3 percent of the European Union's GHG emissions and nearly 70 percent of those come from the animal sector[3]. The most significant element in livestock farming is the animal feed.

The European Commission put forward an Action Plan on organic farming[4], which evidently contributes to more sustainable food production. Furthermore, it works on an Action plan for integrated nutrient management to reduce the pollution from fertilisers.

Three key elements of addressing the role of animal feeding are feed sources, feed additives, feeding systems and feed labelling. Various legal instruments have been developed to address each of these elements.

More sustainable feed sources

Feed materials to be reduced:

- Soybean and palm feed from deforested land.
- Fish meal from unsustainable fisheries.
- Food grade grains.

Feed materials to bolster:

- Seaweed and other feed from the oceans.
- By-products from the food, biofuel and bio-economy.
- Processed animal proteins derived from animals fit for human consumption.
- Insect derived feed materials.

Instruments: Revision of the Catalogue of Feed Materials (Regulation 68/2013) and TSE Regulation (EC) 999/2001

Innovation in more sustainable feed additives

a. *Reducing the negative impact of livestock farming on the environment*

- Climate change mitigation by reduction of GHG (e.g. methane) emission.
- Protection of soil, water and the atmosphere from nutrient losses e.g. reduction of phosphorous and nitrogen excretion from animals (soil leaching and water eutrophication) or less ammonia emissions (particular matter precursor).
- Fostering an efficient use of resources. Additives improving the zootechnical parameters => feed conversion => smaller carbon footprint.
- Additives contribute to reduce feed losses during storage by preservation.

b. *Animal welfare/health benefits*

- Additives stabilising the animals intestinal flora reduce the need for medicinal treatments such as with antimicrobials, thus reducing the risk of antimicrobial resistance.
- Additives improving the physiological status of animals, reducing stress to adverse climate conditions or easing the transition to different production stages in the life cycle enhance animal welfare.

c. *Economic benefits*

- Positive macro-economic impact of innovation in feed additives: R&D investments fuel growth and jobs.
- Livestock farmers can increase their profitability and competitiveness.

Instrument: Revision of Feed Additive Regulation (EC) No 1831/2003

Modern feeding systems

- Reduced emissions by combinations of several feed additives and feed materials, targeted to the specific animal and its life stage.
- Modern feeding technology (such as mixing, fermentation, grinding, and coating) to improve efficiency.
- Optimised feeding by precision and digital farming.

Instrument: Training activities under the Common Agricultural Policy framework

Sustainable feed labelling for informed consumer choices

- Establishment of a methodology for environmental foot-printing (EF) based on life cycle assessment for the complete diets of European Union livestock.
- Voluntary application by feed industry possible => allows upstream foot-printing.
- Draft legislation with options: Any green claim about feed shall be substantiated via a EF methodology.
- Include requirements on communicating EF profiles, enforcement, certification.

Instrument: Upcoming new European Union legislation on Green Claims

3. Conclusions

- Animal feed is the most important element with respect to sustainability of animal products.
- The European Union's legal framework for feed will be further modernised to allow that new feeding concepts and feed ingredients can considerably reduce the negative impacts of livestock farming on the climate and environment.
- These actions in the field of animal nutrition are embedded in the holistic sustainability concept from farm to fork.

[3] EEA (2019), Annual European Union greenhouse gas inventory 1990-2017 and Inventory report 2019

[4] https://ec.europa.eu/info/food-farming-fisheries/farming/organic-farming/organic-action-plan_en

Integrating technologies for the sustainable control of gastrointestinal parasites in sheep: The Argentinean case

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Abstract

Gastrointestinal nematode infections in sheep are a major concern among breeders due to the economic losses they cause in terms of a reduction in both productivity and viability of animals. The situation worsens in face of the emergence of anthelmintic-resistant parasites. In this context, breeding and management practices aimed at an integrated control of parasites, such as raising parasite-resistant sheep, are required. This study focused on the genetic variation underlying parasite resistance in sheep, for potential use in breeding programmes. An artificial challenge with infectious *H. contortus* L_3 was carried out in the northeast region of Argentina for more than 10 years in 1 072 Corriedale lambs with an average age of 5.6 months. Body weight, faecal egg count, packed cell volume, and FAMACHA© score were recorded at different time points post-challenge and their heritability and phenotypic and genetic correlations were estimated. Animals were genotyped on 173 single nucleotide polymorphisms belonging to 77 candidate genes for immune response. The results indicate that there is sufficient genetic variability for the four traits studied, which presented moderate heritabilities (in the range 0.29 to 0.44) and increased along the challenge period, with the exception of the hematocrit, which decreased. Association analyses identified seven markers associated with estimated breeding values for faecal egg count, located in genes involved in different stages of the pathogen-host interaction process. The information obtained supports the potential of marker-assisted breeding schemes to enable profitable and sustainable sheep production.

Keywords

gastrointestinal nematodes, resistance/resilience, variance components, Corriedale sheep, candidate genes

1. Introduction

Gastrointestinal nematodes (GINs) impose severe restrictions on sheep production around the world and are a major cause of economic losses. In the northeast region of Argentina, approximately 2.1 million sheep are raised (14.2 percent of the country's total sheep) in 37 288 productive units (31.6 percent of the total units) and in the central region 2.7 million sheep are raised (18.3 percent of the total for the country) in 45 298 productive units (38.3 percent of the total units). The productive units, mostly made up of family structures, belong to medium and small producers. Breeding is outdoor on natural and/or cultivated pastures (Faverio *et al.*, 2016). The temperature and humidity conditions in both regions are conducive to the development of parasites and the most infective and abundant species is *Haemonchus contortus*.

Although mortality is, in general, the most visible sign of intense parasitism, the loss of body weight in lambs is another consequence, which can reach up to 10 percent. GINs cause reductions of 15–20 percent in wool production, with estimated loss of around USD 2 per animal annually. On average, the death of each reproductive female causes losses of around USD 70 and for each dead ram, USD 400 would be lost (Cetra, B., personal communication).

The common practice for the control of GINs is by means of antiparasitic drugs, which are used several times a year, generally based on the pattern of infestation (Suarez and Busetti, 1995). However, the indiscriminate use of antiparasitic drugs "to clean flocks" without a real notion of key concepts on epidemiology and other complementary measures for control has resulted in the emergence of drug-resistant parasites (Suárez, 2007). Evidence of resistance of parasites to biocidal drugs was shown for the first time in Argentina in the '90s and later, in a study carried out in the province of Corrientes by INTA-FAO (2003–2005), it was shown that 80 percent of the flocks had parasite resistance to all drugs available in the market (Caracostantogolo *et al.*, 2005).

In the Mesopotamian region of Argentina, the resistance of *H. contortus*, and other species, to benzimidazoles has been reported and the increase and geographic dispersion of cases of multiple drug resistance in the central area of the country such as Santa Fe and Buenos Aires provinces are of particular concern (Anziani and Fiel, 2015).

Although new formulations appeared in the market since 2010 (i.e. monepantel and derquantel), after five years, there are already reports of resistance to monepantel in Argentina and Uruguay (Mederos, Ramos and Banchero, 2014; Cerutti *et al.*, 2018). Recently (as of September 2020) the use of the drug Naftalofos (Vermkon, König) was presented and approved in Argentina by SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria – www.argentina.gob.ar/senasa).

The ability of parasites to generate drug resistance is certainly much faster and more efficient than expected. In this "race" for the development of new drugs and their massive and indiscriminate application, the major consequence is not only the creation of new resistant strains, but also the generation of waste that contaminates food and the environment.

There are alternative and complementary practices that aim for integrated control of parasites, such as the use of condensed tannins, the management of pastures and flocks, and a vaccine for *Haemonchus* (developed in Australia – Barvervax). Nonetheless, opportunities to apply such practices are limited to certain geographic regions and some are expensive or cannot be applied due to the type of production system in which the animals are raised. In this context, it is necessary to search for and implement new options for sheep farming in these regions that help to reduce losses, stimulate profitable and sustainable production over time, and minimize contamination with chemicals.

As reviewed by Periasamy *et al.* (2014), there is considerable variation among and within sheep breeds in their ability to resist gastrointestinal nematodes. The most widely accepted and practiced measure of resistance/susceptibility to GIN is the count of eggs per gram of faeces (FEC). This trait, in general, has a low to medium heritability (Assenza *et al.*, 2014; Vanimisetti *et al.*, 2004). The possibility of raising sheep more resistant to gastrointestinal parasites in different production systems was widely discussed, with ample evidence reported by various authors (Vagenas *et al.*, 2002; Miller *et al.*, 2006; Kemper *et al.*, 2010; Bishop, 2012; Karlsson and Greeff, 2012; Atlija *et al.*, 2016; Benavides *et al.*, 2016; Aguerre *et al.*, 2018; Stear *et al.*, 2019; Haehling *et al.*, 2020).

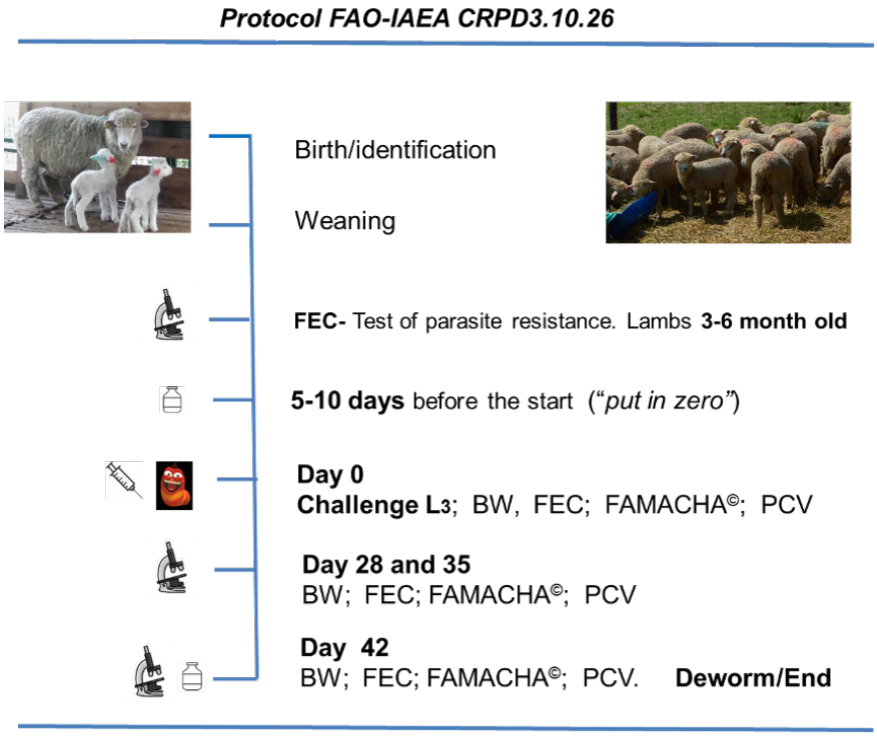
In the year 2009, the INTA´s project AERG-234002 launched a new phase of the former study on parasite resistance in sheep and the following year it was part of a multinational collaborative project sponsored by the International Atomic Energy Agency (IAEA) and the Food and Agriculture Organization of the United Nations through the IAEA Collaborative Research Project Number D3.10.26. The current study is part of this collaborative project.

The main objective of this study was to find genetic variation underlying parasite resistance in sheep to be used in breeding programmes. To accomplish that, the following specific objectives were posed: (i) to estimate variance components for body weight (BW), faecal egg count (FEC), packed cell volume (PCV), and FAMACHA© score (FAM); (ii) to estimate phenotypic and genetic correlations between these traits in Corriedale lambs after an artificial challenge with infective larvae; and (iii) to perform association analyses with a set of single nucleotide polymorphisms (SNPs) within candidate genes for immune response.

2. Materials and methods

Animals, phenotypes and genotypes

Artificial challenges were performed on 1 072 Corriedale lambs of both sexes, from 35 ram half-sib families, with complete pedigree information for three generations. The animals were born between 2010 and 2021. Procedures for challenge experiments and blood sample collection were approved by the Institutional Committee for Care and Use of Experimental Animals (CICUAE) of the National Institute of Agricultural Technology (INTA) and were carried out in strict accordance with the guidelines specified in the institutional manual. Lambs at risk were treated urgently and removed from the trials. The flocks studied belonged to two INTA experimental stations located in the northeast of Argentina. Briefly, the protocol used was as follows (Figure 1): animals were weaned 90 days after birth and kept on field until they were 4 to 6 months of age. At that time, lambs were separated by sex, dewormed, and moved to a pen. Lambs were artificially challenged via the rumen with 5 000 infective third-stage larvae (L_3) of *Haemonchus contortus*. At days 0, 28, 35 and 42 post-challenge, body weight (BW0, BW28, BW35 and BW42, respectively), faecal egg count (FEC0, FEC28, FEC35 and FEC42, respectively), packed cell volume (PCV0, PCV28, PCV35 and PCV42, respectively) and FAMACHA© (FAM0, FAM28, FAM35 and FAM42, respectively) were recorded. Later, animals were dewormed and bred in extensive systems under common flock management. The PCV was determined from total blood by the microhematocrit centrifuge method. Faffa Malan Chart (FAMACHA) score was recorded by examination of the mucous membrane of the eye and comparing it with the five-value scale of the FAMACHA© chart (Bath, Malan and Van Wyk, 1996). It was registered at each time point by the same trained technician on each location to minimize errors.



Source: Authors' own elaboration.

Figure 1: Protocol from CRPD3.10.26 FAO-IAEA

Genomic DNA was obtained from blood samples using commercial kits following the manufacturers' protocols. As a first step of the ongoing project, a total of 173 SNPs belonging to 77 candidate genes for immune response from every ovine chromosome except for chromosomes 4, 9, 10, 18, 21, 23, and Y were genotyped on 624 animals. The second step (in progress) consists in the genotyping of a larger number of animals with SNP microarrays to perform a genome-wide association study. Candidate genes and markers were selected as described in (Periasamy *et al.*, 2014). Genotyping was performed by competitive allele specific PCR (KASPar) assays based on FRET chemistry (KBiosciences, LGC Genomics, UK). Cycling conditions for each assay were those recommended by the manufacturer. BioRad CFX96 (BioRad, USA) software was utilized for genotype calling. A quality control (QC) check of the genotypes was performed using PLINK v1.9 software (Chang *et al.*, 2015). QC consisted in excluding samples with average call rate < 95 percent, as well as removing from the analysis individual SNPs with call rate < 95 percent and minor allele frequency (MAF) < 0.025. The latter parameter was determined using SNP

frequencies on founder animals to avoid bias due to inbreeding. No SNP was highly deviated from Hardy-Weinberg equilibrium (all SNPs showed *p*-values for HWE exact test > 1.10^{-6}).

Statistical analysis

FEC was not normally distributed, consequently, observed values of FEC were log transformed, LNFEC = ln (FEC + 250). Univariate analyses were conducted, using mixed model procedures in LME4 (Bates *et al.*, 2022) package in R software (R Core Team, 2014) to identify effects and covariates that contributed significantly to the variation of BW, LNFEC, PCV and FAMACHA© index. These models included, for all response variables, lamb's BW at the beginning of the trial as a covariate; farm, year of trial, and sex as fixed effects; and ram as a random effect; plus, for each response variable, its value at day zero as a covariate. Based on the selected models, univariate animal mixed models were used to estimate additive genetic variance (and estimated breeding value, EBV) for BW, LNFEC, PCV and FAMACHA© index means (days 28, 35 and 42). Phenotypic and genetic correlations were estimated using bivariate animal mixed models. Restricted maximum likelihood (REML) estimates were obtained with the EM algorithm using the WOMBAT software (Meyer, 2007).

After genotype QC, 623 lambs and 141 SNPs remained in the dataset. Association analyses between each individual SNP and the EBVs for FEC were carried out in PLINK v1.9 using linear models. Additive linear models were fitted and adjusted for significant covariate effects which included sex, farm, challenge age, PCV0, and the first two principal components derived from a principal component analysis performed using PLINK v1.9. To account for the risk of false positives due to the multiple testing problem, *p*-values were adjusted by Bonferroni correction. Corrected *p*-values < 0.05 were accepted to represent a proof of significant associations with the character under study.

3. Results and discussion

Total data reordered, structured and descriptive statistics for age (in days), body weight (BW), average faecal egg count (FEC), ln (FEC+250), package cell volume (PCV) and FAMACHA© traits in the Corriedale sheep are presented in Table 1.

Among South American countries, the most similar breeding conditions in Corriedale sheep grazing systems are those of Argentina and Uruguay. Because the protocols used in Uruguay to record descriptive traits of resistance/resilience to GIN differ in the larval challenge (natural vs artificial) and in the age of the animals, Uruguay's mean values for BW and PCV are higher (34.42 and 35.39, respectively) than in Argentina but conversely, the average values for LogFEC and FAMACHA© are smaller (6.61 and 2.53, respectively) (Ciappesoni and Goldberg, 2018).

A summary of the descriptive statistics for the phenotypic traits on Corriedale lambs at 0, 28, 35 and 42 days post-challenge is shown in Table 2.

The mean BW during the 42 days of challenge increased by 1.5 kg. There were significant differences ($p < 0.001$) between all the contrasts except between day 28 and day 35 ($p > 0.05$). Faecal egg counts increased over time, showing significant differences ($p < 0.001$) between day 0 and the rest of the days and between day 28 vs 35 and 42, and for days 35 vs 42 too. Conversely, the FAMACHA© mean increased over time from 2.7 to 3.4 with significant differences in all contrasts.

Estimated heritabilities, genetic and phenotypic correlations for BW, LNFEC, PCV and FAMACHA© means are shown in Table 3.

On average, heritabilities, genetic, and phenotypic correlations had standard errors of 0.06, 0.15 and 0.03, respectively.

The estimated h^2 were similar for FAM (0.29), PCV (0.31) and LNFEC (0.32) and higher than those reported by Balconi Marques, Goldberg and Ciappesoni (2020) for the same breed in Uruguay (0.10, 0.25 and 0.19, respectively). The h^2 for BW was 0.44 (0.05), higher than those reported by Ciappesoni and Goldberg (2018) and Balconi Marques, Goldberg and Ciappesoni (2020) (0.35 and 0.33, respectively).

The BW was favourably genetically correlated with LNFEC (-0.42) and with FAM (-0.29), however the genetic correlation with PCV was practically null (0.07). Balconi Marques, Goldberg and Ciappesoni (2020) found similar values for BW - LNFEC and FAM, but with a lower magnitude (-0.09 and -0.17).

The negative genetic correlations between PCV and FAM (-0.46) and between PCV and LNFEC (-0.65) and the positive genetic correlation found between LNFEC and FAM (0.76) are indicative of a typical response to the hematophagous parasite *Haemonchus sp*.

The negative genetic correlations between BW and LNFEC (-0.42) and the slightly positive genetic correlation between BW and PCV (0.07) are indicative of resistant and resilience traits.

The FAMACHA© score h^2 (0.29) and its positive genetic correlation with FEC (0.76) suggest it can be used as a reliable indicator of parasitism with *Haemonchus sp*.

The phenotypic and genetic negative correlations between BW and LNFEC and positive correlation with PCV allow selecting animals for both characters simultaneously with a positive response.

The minimum and maximum EBV, estimated with an accuracy of ≥ 0.7, were as follows: BW −2.4 kg to +4.2 kg; FEC -1462 to +2469; PCV +4.06 percent to −2.3 percent and FAMACHA© −0.38 to + 0.50 units.

Table 1: Descriptive statistics for age (in days) body weight (BW), average faecal egg count (FEC), Ln (FEC+250), package cell volume (PCV) and FAMACHA©

Trait	N	Mean	Standard deviation	Maximum	Minimum
Age (days)	1 072	169	20.83	237	105
Body weight (BW; kg)	1 071	23.95	4.97	40.10	11.00
Average FEC (FEC)	1 069	1 845	3 535	17 700	0.00
Ln FEC (LNFEC)	1 069	7.66	0.97	10.09	5.52
Packed cell volume (PCV)	1 062	24.68	5.05	37.50	6.67
FAMACHA© score (1-5)	1 072	3.31	0.74	5.00	1.00

Source: Authors' own elaboration.

Table 2: Descriptive statistics for the phenotypic traits during the 42 days of artificial challenge with L_3 using the CRPD3.10.26 FAO-IAEA protocol

BW Day	n	Average	SD	Max	Min
0	1 071	23.1	5.21	40.0	9.9
28	1 070	24.1	5.16	39.9	10.6
35	1 063	24.2	5.04	43.0	11.3
42	1 059	24.6	5.29	40.6	10.5
FEC Day	**n**	**Average**	**SD**	**Max**	**Min**
0	1 069	0	0.00	0	0
28	1 050	2 460	3 296.85	38 300	0
35	1 045	3 644	4 163.20	28 400	0
42	1 056	4 599	6 227.50	58 700	0
PCV Day	**n**	**Average**	**SD**	**Max**	**Min**
0	1 062	27.8	4.63	42	9
28	1 056	25.2	4.99	38	7
35	920	24.7	5.45	42	7
42	1 047	24.2	5.52	38	5
FAM Day	**n**	**Average**	**SD**	**Max**	**Min**
0	1 072	2.7	1.06	5	1
28	1 068	3.1	0.92	5	1
35	1 061	3.3	0.85	5	1
42	1 056	3.4	1.07	5	1

Source: Authors' own elaboration.

Table 3: Heritabilities, genetic and phenotypic correlations, and their standard deviations

Trait	BW	FAM	PCV	LNFEC
BW	**0.44** (0.05)	-0.29 (0.13)	0.07 (0.15)	-0.42 (0.12)
FAM	-0.36 (0.03)	**0.29** (0.06)	-0.46 (0.12)	0.76 (0.10)
PCV	0.20 (0.04)	-0.50 (0.03)	**0.31** (0.07)	-0.65 (0.12)
LNFEC	-0.29 (0.05)	0.42 (0.03)	-0.50 (0.03)	**0.32** (0.06)

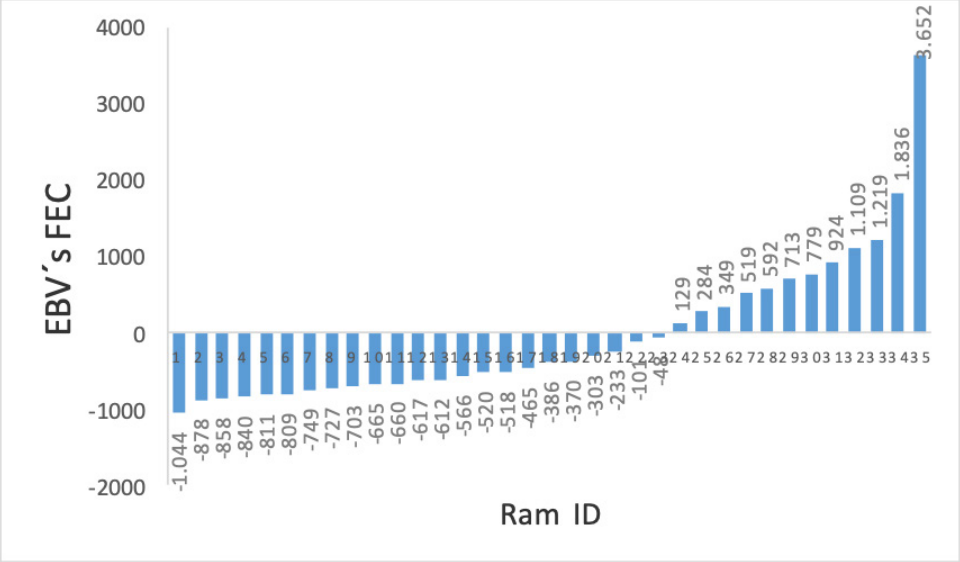
Source: Authors' own elaboration.

Note: Heritabilities on the diagonal, genetic correlations above the diagonal and phenotypic correlations below the diagonal. Standard deviations between brackets.

Figure 2 shows the distribution of FEC EBVs for the 35 Corriedale rams with at least 10 progeny. These EBVs are relative to the mean of FECs after back-transforming LNFEC; in 2020, that mean was equal to 3092. The maximum and minimum FEC EBVs values observed in rams were 3 652 and -1 044 respectively. EBVs are indicators of the genetic merit of each ram and can be used to predict future changes for FEC of their progenies (EPDs).

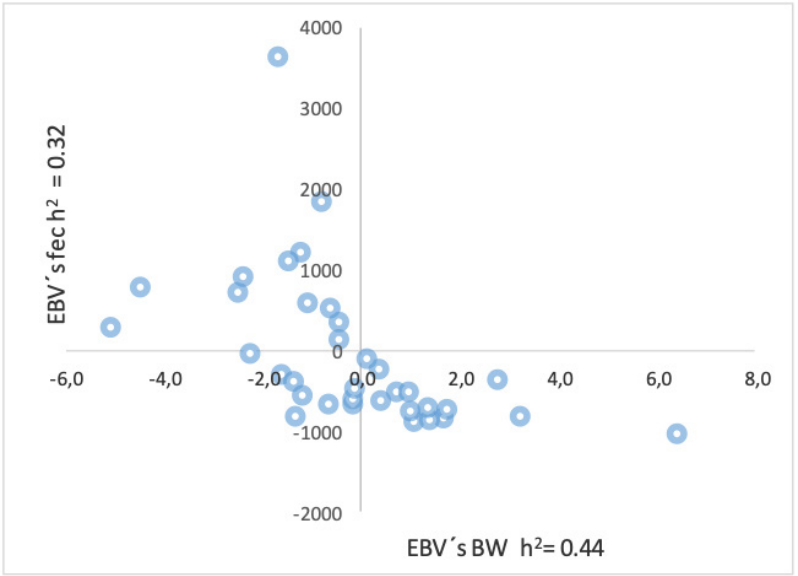
Figure 3 shows the distributions of FEC and BW EBVs for the 35 rams. We found that 37 percent (13/35) of the rams had negative values for FEC EBV and positive values for BW EBV.

Seven SNPs showed significant corrected *p*-values < 0.05 when testing association to FEC EBVs (Table 4). Among those significant associated markers, we found that OLADRA1_479, a SNP in the MHC-Ovine Lymphocyte Antigen DRA gene on OAR20, showed the lowest *p*-value. Then we found four SNPs on OAR3 (in decreasing order of significance: CLEC12A_567, CLEC8A_532, CLEC12A_440, and IL2RB_180). Those SNPs were located in two genes of C-type lectin domain families and in the Interleukin 2 receptor β gene. The last two SNPs associated with FEC EBVs obtained in this study and adapted from Raschia *et al.* (2021) were TLR10_292 and MASP2_104, on sheep chromosomes 6 and 12, respectively, which corresponded to the Toll-like receptor 10 and the Mannan binding lectin serine peptidase 2 genes (Table 4).



Source: Authors' own elaboration.

Figure 2: FEC EBVs distribution for 35 Corriedale rams



Source: Authors' own elaboration.

Figure 3: BW and FEC EBVs distribution for 35 Corriedale rams

Table 4: SNPs associated to FEC EBVs

SNP	Position (OAR:bp) [1]	Candidate gene	Bonferroni-corrected *p*-value
OLADRA1_479	20:25775019	*OLA-DRA*	9.21 e-7
CLEC12A_567	3:204592787	*CLEC12A*	0.001683
CLEC8A_532	3:204456657	*CLEC8A*	0.001752
CLEC12A_440	3:204592660	*CLEC12A*	0.002115
IL2RB_180	3:180362559	*IL2RB*	0.002177
TLR10_292	6:57993319	*TLR10*	0.002937
MASP2_104	12:40735932	*MASP2*	0.01938

Source: Authors' own elaboration.

Significant SNPs found to be associated with FEC EBV are located in genes involved in different stages of the pathogen-host interaction, such as pathogen recognition (*TLR* and C-type lectin domain gene families), innate immune response (*MASP* and *TLR*), and adaptive response to infection (C-type lectin domain gene families, *IL2RB*, and *OLA-DRA*).

4. Final remarks

Gastrointestinal nematodes in sheep have rapidly become resistant to all drugs developed in recent years and there is no doubt about the need for integrated management strategies for the control of parasites. In addition to pasture rotation, deworming strategies involving FAMACHA© score, alternative antiparasitics (i.e. tannins), among other practices, the use of genetically more resistant and resilient sheep is envisaged as the most sustainable and "clean" strategy over time.

The CRPD3.10.26 FAO-IAEA protocol used for more than 10 years allowed us not only to obtain phenotypic and genotypic information for a breeding programme in the Corriedale breed by having the components of variance, correlations and heritabilities, but also to find the genetic variability underlying GIN resistance and resilience traits in sheep. Furthermore, none of the challenged animals was negatively affected neither for growth nor for health and the artificial challenge with L_3 allowed us in a short time, 35–40 days, to obtain data on resistance and resilience to GIN.

The results indicate that the Corriedale breed has sufficient genetic variability for the four traits studied and the values of heritability and genetic and phenotypic correlations obtained support a potential use for genetic progress in all traits.

The high genetic correlation between FEC and FAMACHA© estimated in this work (0.76) and the negative genetic correlation between FEC and PCV (-0.46) suggest the possibility of using both traits to select animals for resistance and resilience to GIN.

The protocol presented in this study requires repeated measures, however. An alternative could be to do only one FEC and FAMACHA© score determination at day 35. The correlation found between the average of FEC28, FEC35, and FEC42 and FEC on day 35 was 0.93 in this study.

Currently, the protocol is being used in four experimental units and in two private stud flocks in Corriedale, Texel and Ideal breeds.

By means of the association analyses performed using the candidate gene approach, we identified single SNPs that had a significant association with nematode resistance in Corriedale sheep. This information has potential use in marker-assisted breeding schemes of Corriedale sheep in Argentina, which constitute a promising long-term strategy to effectively reduce parasitic infections and enable a profitable and sustainable sheep production. Nonetheless, we should further explore the variability in loci not included in this study to have a clearer picture of the genetic regions underlying GIN resistance in sheep.

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Growth performance of Yemeni sheep and their Awassi crosses

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Abstract

This study evaluates the growth performance of purebred Yemeni and crossbred Awassi (½ Awassi, and ¾ Awassi crossbred) sheep. The data was obtained from eight consecutive years of records from the Regional Research Station in the Central Highlands of Yemen. The recorded data included genetic group, the weight of dam at lambing, year and season of birth, sex, birth weight (BW), weaning weight (WW), average daily gain (ADG), and survival from birth to weaning. BW and WW were significantly different among the genetic groups ($P < 0.001$). The BW and WW of ¾ Awassi crossbred lambs were higher than both purebred Yemeni lambs and ½ Awassi lambs ($P < 0.05$). The superiority of crossbred lambs (½ Awassi, ¾ Awassi) over purebred Yemeni sheep from birth to weaning ranged between 24 percent and 33 percent. Likewise, the average daily gains in ¾ Awassi and ½ Awassi lambs were significantly ($P < 0.05$) greater than Yemeni sheep, at 142.89, 133.20 and 110.31 gm/day, respectively. Genotypes of lambs had a significant effect ($P < 0.05$) on their survival. The survival rate during the early stages of life was greater for purebred Yemeni lambs, whereas the crossbred lambs showed superior survival at advanced ages. The Awassi breed could be appropriate for crossbreeding to improve the productivity of Yemeni sheep.

Keywords

Yemeni sheep, purebred, growth performance, crossbred

1. Introduction

Sheep contribute meat, wool and skin as well as manure and serve as a sole or subsidiary source of income to the livelihoods of a large number of small-scale and marginal farmers and landless laborers in Yemen. There are 11 recognized traditional sheep breeds in Yemen, of which five are wool and six are hair types (Wilson, 2003). All sheep breeds in Yemen are indigenous and are fat-tailed, although the conformation of the tail varies considerably (Hasnain, Nokhie and Iryani, 1994). According to the 2019 agricultural census, there were 8.8 million sheep in Yemen and they represent about 45.4 percent of the total livestock population. The local sheep, despite their poor growth rates and low prolificacy, are characterized by the ability to survive and reproduce in the rangeland without supplementary feeding (Albial and Singh, 2013). Awassi sheep (well-known meat sheep in Syria) have better growth rates than purebred Yemeni sheep. A crossbreeding programme has been conducted by using improved Awassi males in an artificial insemination programme to improve the body weight of purebred Yemeni sheep. Hence, this study aimed to evaluate the growth performance of crossbreds of Yemeni ewes with improved Awassi males (½ Awassi, and ¾ Awassi crossbred) at different ages.

2. Materials and methods

Flock management practices

The sheep involved in this study were purebreds of Yemeni sheep and crossbreds of purebred Yemeni sheep with improved Awassi males (½ Awassi and ¾ Awassi) obtained by using artificial insemination in cooperation with the Arab Center for the Studies of Arid Zones and Dry Lands (ACSAD). The flocks were raised at the Regional Research Station in the Central Highlands, Dhamar, Yemen. The management system used at the station was similar to the traditional system of sheep husbandry in the area,

in terms of grazing and diet supplementation. All ewes grazed together during the day for about 6 to 8 hours. When not grazing, they were housed in covered pens with free access to grass hay, water and mineral lick blocks. A daily supplementary concentrate ration of 250 to 500 g/head, depending on season and physiological status, was fed to animals in the morning before grazing. A controlled mating scheme was used with three mating periods over two years. After lambing, each ewe was put separately with its lamb into a lambing pen for about 2 to 7 days. Ewes and their lambs were weighed and ear-tagged 24 hours after lambing. The weaning of lambs took place at an average of 91 ± 8 days. Lambs were weighed again at weaning, which allowed for the calculation of average daily gain (ADG). Mortality was recorded throughout the study period.

Statistical analysis

The data utilized in this study were 552 and 502 records of weights of lambs at birth and weaning, respectively, from 2013 to 2020. The impact of factors affecting the growth performance of lambs, such as genotype, lamb sex, and weight of dams at lambing, and the season and year of lambing was investigated using the least-square procedure (SAS, 2003). Duncan's test was used to determine differences among subgroups means. The analysis was performed using the following linear model:

$$Y_{ijlmdgr} = \mu + B_i + S_j + A_l + C_d + G_r + e_{ijlmdgr}$$

Where: $Y_{ijlmdgr}$ is the studied trait, μ overall mean, B_i the fixed effect of the ith genotype (Purebred Yemeni sheep, ½ Awassi, or ¾Awassi), S_j the fixed effect of the jth sex of lamb (male or female), A_l the fixed effect of the lth weight of dam at lambing (1, 2 or 3) where 1 = ≤ 25 kg, 2 = 25 to 34 kg and 3 = ≥ 35 kg, C_d the fixed effect of the dth season of lambing (summer, autumn or winter), G_r the fixed effect of the rth year of lambing (from 2013 to 2020), $e_{ijlmdgr}$ the random error associated with each observation.

3. Result and discussion

Body weights

Least-square means and the corresponding standard errors of body weights of purebred Yemeni lambs, ½ Awasi and ¾ Awassi crossbred lambs are presented in Table 1. The variation in body weights among genotype groups was statistically significant ($P < 0.001$) at birth and weaning. Birth weight and weaning weight of ¾ Awassi crossbred lambs were greater than for both purebred Yemeni and ½ Awassi lambs ($P < 0.05$). The superiority of crossbreed lambs (½ Awassi and ¾ Awassi) over purebred Yemeni sheep from birth to weaning ranged between 24 percent to 33 percent. Better performance of the crossbreds compared with the purebred lambs was presumably due to both the genetic differences between the two breeds and heterosis. Similar results were reported by Dawson, Carson and McClinton (2002) and Momani Shaker *et al.* (2002), who found that crossbreds had higher live weights at birth and weaning and faster growth compared with purebred lambs. Males were significantly ($P < 0.05$) heavier than females at both birth and weaning. The average weights of males exceeded those of females by 3 percent to 7 percent at birth and weaning, respectively. The size of the ewe, which was reflected by dam weight during lambing had also a significant effect ($P < 0.01$) on the birth weight of the lamb for all genotypes. This study showed that lamb birth and weaning weights tended to increase as the live weight of the dam increased from ≤ 25 kg to > 35 kg. This result is in agreement with Dixit, Dhillon and Singh (2001), who reported that an increase in the dams' weight at lambing by 1 kg resulted in a significant increase in body weight at birth by 29 g. The significant effect of the dam's body weight at lambing on its lamb's weight at birth indicated that ewes with higher body weight at lambing provide better prenatal environment for the lamb's fetal growth (Juma, Alkass and Fahim, 2000). Birth weight was affected by the year of lambing but there were no significant differences by lambing season, whereas the lambs born in summer and autumn had higher weaning weight than those born in winter due to the differences in availability of pasture and fodder during the seasons. This finding was in agreement with Albial and Singh (2013) for White Boni sheep and by Thiruvenkadan *et al.* (2010) for Mecheri and their crossbred lambs.

Average daily gain

The ADG from birth to weaning of crossbred lambs was greater than for purebred Yemeni lambs (Table 1). The ADG was 142.89, 133.20 and 110.31 g/day for ¾ Awassi, ½ Awassi and purebred Yemeni lambs, respectively. Similar findings on the effect of genotype on ADG were reported by Marzouk and Mousa (1998) and Thiruvenkadan *et al.* (2009). Male lambs had significantly ($P < 0.05$) higher ADG compared to females (130.49 vs. 119.45 g/day) from birth to weaning. These results are in disagreement with Abbas *et al.* (2010) and Marzouk and Mousa (1998), who found that the effect of sex on ADG was not significant. Weight of dam at lambing significantly ($P < 0.05$) affected growth rate from birth to weaning. Similar positive relationships were reported by Dixit, Dhillon and Singh (2001) and Babar, Ahmad and Nadeem (2004). This may be attributed to the positive correlation that exists between the pre-weaning ADG of the lambs and the amount of milk produced by their dams (Danso *et al.*, 2016), which depends on weights of ewes and their nutrition during the period of pregnancy and suckling. The growth of lambs born during summer and autumn was higher than those born in winter. This may be due to the increased availability of good quality fodder for the dams during the rainy season (summer and autumn) than the winter season. Similar results were also observed by Albial and Singh (2013).

Lambs survival rate

Results for survival rate from birth to weaning are presented in Table 2. Genotypes had a significant effect ($P < 0.05$) on the survival rate of young lambs. The survival rate of purebred Yemeni sheep lambs was significantly higher during the early stages of life. The higher survival rate of Yemeni purebred lambs during birth could be due to their greater adaptation to the local conditions than exotic genotypes. In contrast, crossbred lambs had a (non-signficantly) higher survival rate from birth to weaning. The higher survival rate of crossbred lambs during this period may be due to their heterosis and other factors that also contributed to greater growth rate. The effect of genotype on survival rate is inconsistent with Morsy (2002) and Abd-Allah (2005) who reported that the breed group had a non-significant association with survival rate. On the contrary, El-Karim (1993) and Hamdon (1996) stated that the breed group had a significant effect on survival rate, especially at early ages.

Table 1: Least-square means ± standard error of some factors affecting the growth performance of purebred and crossbred lambs

Fixed effect	Birth weight (kg)		Weaning weight (kg)		Average daily gains (gm)	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
Genetic group		***		***		***
Purebred (Yemeni sheep)	299	2.49 ± 0.06 [c]	252	12.45 ± 0.45 [c]	251	110.31 ± 4.74 [c]
½ Awassi	65	3.43 ± 0.06 [b]	63	15.56 ± 0.52 [b]	60	133.20 ± 5.59 [b]
¾ Awassi	188	3.61 ± 0.07 [a]	187	16.47 ± 0.44 [a]	175	142.89 ± 4.67 [a]
Sex		***		***		***
Male	271	3.08 ± 0.06 [a]	246	14.99 ± 0.44 [a]	239	130.49 ± 4.72 [a]
Female	281	2.96 ± 0.06 [b]	256	13.88 ± 0.44 [b]	247	119.45 ± 4.64 [b]
Weight of dam		***		***		***
≤ 25 kg	147	2.58 ± 0.07 [c]	124	12.80 ± 0.47 [c]	123	114.67 ± 5.02 [b]
24 – 34 kg	264	2.94 ± 0.06 [b]	243	13.99 ± 0.45 [b]	233	119.79 ± 4.80 [b]
≥ 35 kg	141	3.63 ± 0.07 [a]	135	17.00 ± 0.46 [a]	130	143.62 ± 4.93 [a]
Season of birth		n.s		***		***
Summer	286	3.02 ± 0.06 [a]	255	14.84 ± 0.44 [a]	255	131.10 ± 4.64 [a]
Autumn	150	2.91 ± 0.07 [a]	131	14.34 ± 0.48 [a]	130	126.45 ± 5.10 [a]
Winter	116	2.99 ± 0.07 [a]	116	13.23 ± 0.46 [b]	101	107.09 ± 4.98 [b]
Year of birth		***		n.s		n.s
Period 1 (2013 - 2016)	280	3.04 ± 0.06 [a]	242	14.53 ± 0.44 [a]	241	124.08 ± 4.67 [a]
Period 2 (2017- 2020)	272	2.92 ± 0.06 [b]	260	14.13 ± 0.44 [a]	245	125.64 ± 4.71 [a]

Source: Authors' own elaboration.

Notes: a n.s: Non-significant.

b * Significant ($P < 0.05$), ** Significant ($P < 0.01$), *** Significant ($P < 0.001$).

c [a-c] Means with different letters in each subclass within a column differ significantly at ($P < 0.05$).

Table 2: The effect of genotype on the survival rate of purebred and crossbred lambs

Fixed effect	Survival rate %			
	n	At birth	*n*	At weaning
Overall mean	594	93.78	577	95.85
Genetic group		*		**n.s**
Purebred (Yemeni sheep)	324	97.52[a]	309	94.50
½ Awassi	69	93.76[b]	69	98.56
¾ Awassi	201	91.67[b]	199	96.99

Source: Authors' own elaboration.

Notes: a [a,b] Means with different letters in each subclass within a column differ significantly at ($P < 0.05$).

b *n* Means number of records.

4. Conclusions

Careful choice of prolific breeds and appropriate crossbreeding can significantly contribute to an increase in mutton production. In this study, ½ Awassi and ¾ Awassi crossbred sheep had better growth performance than purebred Yemeni sheep. This result may presumably indicate the effect of hybrid vigor on first- or second-generation crosses of ½ or ¾ Awassi blood compared with pure Yemeni sheep. A genetic superiority in body size and growth between the Awassi and Yemeni sheep breeds may also explain part of the difference. We conclude that the Awassi breed could be an appropriate breed for crossbreeding to improve Yemeni sheep productivity and early lamb growth in the Yemen production environment.

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Genetic improvement programmes in Nepal: Current status and way forward

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Abstract

Genetic improvement of farm animals has been a prime concern over the years for breeders the world over. Many pedigree selection programmes have been implemented using performance recording schemes to select outstanding individual animals to improve the production and productivity of the respective target populations. Similarly, assisted reproductive technologies like artificial insemination, sexed semen, embryo transfer have been employed to increase selection intensities and reduce the generation intervals in farm animals. The main objective is for each animal to be of high genetic merit and productivity throughout its life to yield maximum profits to farmers. The progress achieved during the recent few years through the combination of pedigree selection programmes and assisted reproductive technologies, especially artificial insemination, has been phenomenal in certain livestock populations. However, as in other developing countries, implementing a pedigree selection programme in Nepal has always been a challenge in the village herds of smallholder farmers. Lack of animal identification and the absence of a system for recording and analysing performance data to make appropriate breeding decisions are the main constraints that have limited the enhancement of animal productivity by breeding. Advancement in modern biotechnologies such as assisted reproductive technologies, genomics and nuclear techniques will play an important role in the future prospective and vision that will increase productivity of farm animals in Nepal.

Keywords

assisted reproductive technologies, genomics and nuclear techniques

1. Introduction

Nepal is predominantly an agrarian country where livestock is an integral and important component of mixed farming systems. Livestock plays a crucial role in providing food (meat, milk, eggs) for human consumption, manure for maintaining soil fertility, power for agricultural operations, and wool and hides for cottage industries. Small ruminants (sheep and goats), yak and equines are also important means of transport in the inaccessible rural areas. Besides, the animals also have sociocultural significance. Nepalese farmers regard livestock, particularly small ruminants and non-ruminants, as "living banks" to provide a source of cash at the times of crisis. Around 27 percent of the Agricultural Gross Domestic Products and around 12 percent of National Gross Domestic Products are contributed by livestock in the country (MoAlD, 2019). Around 75 percent of households keep cattle, nearly 50 percent of the households keep buffaloes and more than 50 percent have goats. The populations of cattle, buffaloes, sheep, goats, pigs and poultry are 7.2 million, 5.2 million, 0.8 million, 9.8 million, 1.16 million and 48 million respectively (SINA, 2013). Dairy farming is the most important livestock activity, accounting for 62.7 percent of the total livestock sector value added, followed by meat (32.4 percent) and eggs (5.0 percent). Geographically,

over half of the cattle, buffalo, goats and sheep are being maintained in the hills, and about one third in the Terai. The native breeds of these livestock species still contribute significantly to the Nepalese economy; exotic breeds and their crosses with native animals are gaining popularity due to population growth, income increases and urbanization, which increase demand for food from animal origin. For a few decades, crossbreeding between indigenous and exotic breeds has been considered to be the best approach for obtaining an exponential increase in productivity of livestock while retaining their adaptability to harsh environments and tolerance to tropical diseases. Crossbreeding technology does not always give satisfactory results, however. The present paper collects information about different genetic improvement programmes of farm animals including assisted reproductive technologies that have been used in the Nepalese context and draws some recommendation on the way forwards.

2. Selective breeding for genetic improvement

Selective breeding is one of the best and most cost-effective tools for genetic improvement of livestock breeds for bringing improvement in the genetic potentials of individual within a population. In Nepal, various initiatives have been taken to improve the genetic potential of livestock breeds of different species mainly including cattle, buffalo, sheep, goat, pigs and chicken (Pokharel and Neopane, 2006; Shrestha *et al.*, 2013; Gorkhali and Bhusal, 2015; Devkota, 2018; Bhattarai *et al.*, 2020; Sapkota *et al.*, 2020). The literature suggests that, when selection is done over many generations, it results in improvement of productive and reproductive traits of livestock species. Genetic improvement programmes in Nepal have played a pivotal role in catering to the increased demand for milk, meat and eggs (Shrestha *et al.*, 2013). It has been reported that egg production and 24-week body weight of indigenous *Sakini* chickens could be increased by more than two-fold by administering selective breeding under optimum environmental management conditions (Sapkota *et al.*, 2020). Animal breeding policies and strategies of Nepal have given due focus on applying selective breeding within the populations of Lime, Parkote and Gaddi buffaloes; Lulu, Achhami, Khaila and Yak/Nak cattle; Baruwal, Bhyanglung, Kage and Lampuchhre sheep; Chyangra and Sinhal goats; and other indigenous breeds for pigs and poultry species.

3. Genetic improvement through crossbreeding

Crossbreeding is the process of mating two or more breeds with the intention to create offspring that share the traits of both parent lineages or to produce animals with hybrid vigour. Crossbreeding allows the improvement of standard traits such as egg production, milk production, growth rate and production of total animal protein. Unpredictable results might be obtained, however, if the genetic merit of the animals and the production system are not considered together. In Nepal, crossbreeding was introduced in 1960 in a small capacity in cattle (Galukande *et al.*, 2013).

Various research findings have concluded that crossbred cattle with 62.5 percent blood level of Jersey have increased productivity in terms of milk while retaining their adaptability to the local farming system (Galukande *et al.*, 2013). On the contrary, the purebred Murrah buffalo breed has exhibited good adaptability in the Nepalese mid-hill and Terai environments (Shrestha *et al.*, 2013). Chickens and goats are the species that show high adaptation potential in different agro-ecological zones; many exotic breeds were introduced and most of them exhibited hybrid vigour when crossed with indigenous populations (Opoku-Mensah, 2017). The results of experimental crossbreeding of Nepalese goats with Jamnapari, Sirohi/Ajmeri, Boer and other breeds suggest that their derivatives would give a better result with respect to higher growth rate and higher meat per doe per annum than the purebred local breed (Bhattarai *et al.*, 2020).

4. Assisted reproductive technologies (ART)

In recent years, livestock productivity has been increased by improved reproduction. Various biotechnologies and techniques have been developed and refined to obtain large numbers of offspring from genetically superior animals or obtain offspring from seemingly infertile animals. These assisted reproductive techniques (ART) include: artificial insemination (AI), cryopreservation of gametes or embryos, induction of multiple ovulations, embryo transfer (ET), *in vitro* fertilization (IVF), sex determination of sperm or embryos, nuclear transfer and cloning. These technologies have been introduced to overcome reproductive problems, to increase the number of offspring from selected females and to reduce the generation intervals in farm animals. Demand and adoption of AI followed by complementary ART have been increasing day-by-day among the dairy cattle and buffalo entrepreneurs (NABGRC Annual Report, 2019). Local scientists have strongly recommended adopting ARTs for improving dairy cattle/buffalo identification, herd health monitoring systems and anoestrus management (Sapkota *et al.*, 2016).

In Nepal, the progress achieved during the last few years in ARTs has been particularly phenomenal in cattle. Artificial insemination is the most effective method being used for enhancing the genetic improvement of animals. Reproductive capacity and efficiency have been improved tremendously since the introduction of AI. Embryo

transfer technology was introduced in 2000, using frozen embryos of New Zealand Jersey cattle (Devkota, 2018). Hitherto ET is done only by the government entities to produce purebred animals of high genetic merit. Proper selection of donor and recipients and application of ART followed by induction of multiple ovulation and ET was reported as an effective technology for enhancing reproductive efficiency of dairy cows (NABGRC Annual Report, 2000). These successful reproductive technologies need be applied on a large scale in Nepal to take full advantage of the opportunities they offer.

5. Genetic selection including assisted reproductive technologies

Performance recording is a prerequisite for effective decision making on breeding policy. The current national dairy cattle improvement project combines programmes for pedigree performance recording and application of ART. It's a collaborative programme between research (National Animal Breeding and Genetics Research Centre) and extension institutes (National Livestock Breeding Organization) and is led by the research institute (Bajagai, 2013). This programme was implemented in well-managed farms where animals are usually identified individually with plastic tags that each display a unique identity number. The aim of the project is to produce high milk yielding dams and genetically superior sires of Nepalese Jersey and Nepalese Holstein within the country. For its first two years this project was implemented as a Technical Cooperation Project (TCP) of FAO and was considered as one of the country's most successful TCPs. After completion of the TCP, the Government of Nepal gave high priority to the programme and has named the National Livestock Breeding Organization as the leading organization. A similar project on buffaloes, on the other hand, was not as successful due to various reproductive problems such as silent heats and low conception rates. Furthermore, buffalo meat is very popular in Nepal, and it is often more profitable to slaughter animals that do not conceive than to wait for the next breeding season.

Earlier, pedigree recording and genetic selection for small ruminants on smallholder farmers had been deemed infeasible by researchers and development workers. Due to uncontrolled breeding practices, it was almost impossible to identify the sire. However, community-based breeding programmes for small ruminants have addressed this problem and have been successful worldwide (NABGRC Annual Report, 2009; Gizaw *et al.*, 2014). Nepal has therefore introduced a pilot community-based goat improvement programme, which was initiated from 2013 by the Ministry of Agriculture and Livestock Development (MoALD). The financial support for this programme has come from the World Bank through the Agriculture and Food Security Project (AFSP) and the International Fund for Agricultural Development (IFAD) through the Improved Seeds for Farmers Program (Karnuah and Dunga, 2018). The main goals of these projects were to improve the non-descript populations by crossbreeding with Boer goat while at the same time improving the identified indigenous goat breeds through breeding for genetic merit. This programme centralized the breeding scheme imposed by the government in order to improve the national goat population. The experience so far indicates that the Nepalese government and the private sector need to invest in complementary activities and infrastructure around community-based goat improvement programmes to make the programme work for the poor and be sustainable in low-input systems (IFAD, 2020). Most of the local governments have implemented projects on improvement of productivity of goats based on the same strategy. Furthermore, a community-based goat breeding programme was launched by the Nepal Agricultural Research Council in the western hills and the project has been funded by USAID under its Livestock System Innovation Lab since 2020.

In all the above cases, pedigree selection has been employed together with ARTs, specifically AI. This combination should help breeders to use selected sires and dams to produce high genetic merit progeny. In these ongoing projects, community-based performance and pedigree recording will be evaluated in terms of the reliability and accuracy of estimated breeding values and genetic parameters and genetic trends across generations. To further address these challenges, the government may consider possibilities to generate genomic data of performance-recorded animals, as this may enable breeders and farmers to relate production traits with parentage and genetic admixture of animals, thereby allowing them to identify and select superior animals for breeding, even before they have performance data.

The lack of animal identification and the absence of a system for recording and analysing performance data to make appropriate breeding decisions are the main constraints that have limited the enhancement of animal productivity by breeding in Nepal. Even in the development government farms, nucleus herds are maintained in the traditional way (i.e. without routine data collection), which has led to deterioration of the breeding stock without a robust breeding programme. Prior implementing advanced technologies on these farms, a system for animal registration needs to be put in place.

6. Nuclear and nuclear derived technologies

The intensification of livestock production systems is a prerequisite to meeting the growing demand for animal products while efficiently using natural resources. Genetic selection, ART and optimized reproduction involving

nuclear and nuclear-derived technologies support the breeding of animals that are more productive while retaining their ability to cope in harsh environments (KUBK Report, 2019). Improving native breeds through genetic selection in a way that maintains their adaptability to local environments and their innate tolerance to local diseases is crucial in addressing the challenge of supplying a persistently growing demand for food of animal origin. Various nuclear and nuclear-derived technologies exist to support such genetic selection procedures.

Radioimmunoassay (RIA) of hormones in milk, blood and other body fluids using iodine-125 is a mature and often used nuclear technique that can be easily performed in decentralized laboratories. It provides unique support in monitoring and improving the outcome of AI services, which could ultimately yield economic benefits to farmers (FAO/IAEA, 2016). In Nepal however, RIA is currently used only in the research institute.

Nuclear techniques can also improve genetics. Irradiation with cobalt-60 can be used to construct radiation hybrid panels for the mapping of livestock genomes. High resolution radiation hybrid maps facilitate genome assembly by correctly ordering genes and genetic markers along chromosomes. Radiation hybrid mapping can be used along with genomic and bioinformatic tools to ultimately support whole genome mapping, which assists the breeders to identify genes affecting their preferred traits.

7. Conclusions

Farm animal selection and improved reproduction benefit greatly from the application of biotechnologies. Biotechnologies such as AI, and multiple-ovulation ET can be powerful tools for rapid genetic improvement of the animal populations. Nuclear techniques also help in breeding (Khanal and Munankarmy, 2009). Radiation hybrid mapping has created an opening of a fascinating scientific arena, especially when applied with genomics and bioinformatics. In Nepal, these advanced reproduction technologies will definitely play an important role in the future perspective and visions for efficient reproductive performance in livestock.

8. Recommendation

A 70 percent increase in the consumption of animal-source protein as food is expected by 2050 due to population growth, income increases and urbanization (FAO, 2011). Consequently, the world will need to increase livestock production manifold. The following ways forward can be recommended to catch up to the ever-rocketing demand by bringing positive changes in conventional breeding.

- Since Nepal is currently using RIA in research only, its application in conjunction with AI should be expanded to the field, to help farmers in detecting non-pregnancy in the early stage.
- Nuclear and nuclear derived techniques for genetic characterization of animals and identification of genetic markers that trace inheritance of favourable traits from parents to offspring should be applied more widely.
- Genomic tools should be applied to improve livestock productivity by determining breed composition of crossbred animals, verifying purity of purebred animals and matching production environments with appropriate genetics to select superior stocks for breeding.
- Farmers should be supported to improve productivity through better access to information about animals' performance.
- Standardized animal identification and data recording procedures should be put in place for implementation of genetic improvement programmes.

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An analysis of genetic diversity and population structure of South African smallholder dairy cattle herds using SNP markers

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Abstract

Information on the genetic makeup of the current cattle population is a prerequisite to the development of a sound and sustainable breeding programme for the smallholder dairy production system in South Africa. Thus, this study was carried out to assess the genetic diversity and structure of the South African smallholder dairy (SHD) cattle population. A total of 189 animals, randomly sampled from twenty-one (21) SHD dairy herds, were genotyped using the GeneSeek® Genomic Profiler 150K-BeadChip. Three specialised dairy breeds, Ayrshire (AYR), Holstein (HOL) and Jersey (JER); and one indigenous breed, Nguni (NGI); were used as reference populations. After quality control, the genotypic data were analysed for genetic diversity and population structure. Expected heterozygosity (H_E) values ranged between 0.39 for JER and NGI to 0.40 for AYR, HOL and SHD. On the other hand, the observed heterozygosity (H_O) values were 0.39 for the NGI and SHD and 0.40 for the rest of the populations. The inbreeding coefficient was slightly lower in the reference populations (-0.004 to -0.02) than in the SHD herds (0.02). Principal Component Analysis (PCA) revealed three distinct clusters that separated the NGI and AYR breeds from other populations. The PCA results concurred with the structure analysis, which showed the SHD population as being predominantly an admixture of HOL and JER. These results indicate widespread crossbreeding in the SHD population, mainly involving HOL and JER breeds, without using NGI and AYR. The information obtained in this study provides a good basis for evaluating the performance of the different admixture levels to develop guidelines for a structured crossbreeding programme for the smallholder dairy production system in South Africa.

Keywords

dairy cattle breeding, genetic improvement, smallholder farmers, single nucleotide polymorphisms

1. Introduction

Poor animal productivity is a major concern in the smallholder dairy farming sector of South Africa (Abin, Visser and Banga, 2018; Bovula *et al.*, 2021). Recent research has highlighted a lack of genetic improvement programmes as one of the biggest factors militating against improved livestock productivity on South African smallholder dairy herds (Bovula *et al.*, 2021). Due to a lack of knowledge of the performance of different breeds in this environment, farmers resort to poorly adapted exotic breeds or unsystematic crossbreeding. Some of them use specialised exotic dairy cattle breeds such as the Holstein (HOL) (Muntswu, Banga and Norris, 2016), despite their well-known impaired performance in low input environments in tropical and sub-tropical regions (FAO, 2015).

Successful efforts have been made in tropical and sub-tropical regions to improve dairy cow performance through grading up of local cattle with improved indigenous breeds, selection within the indigenous breeds, and crossbreeding of native cattle with temperate dairy breeds (Chawala *et al.*, 2021). Such breeding strategies need to be guided by well-informed decisions for breeding replacement stock, based on evaluating different genotypes available in the environment of interest (Marshall *et al.*, 2011; Mrode *et al.*, 2019).

In an admixed population, it is essential to determine the genetic contribution of the parental breeds to available genotypes prior to understanding their performance in the local environment. Molecular markers, particularly single nucleotide polymorphisms (SNPs), have recently been applied successfully to determine the genetic composition in smallholder dairy herds of developing countries (Strucken *et al.*, 2017; Chagunda *et al.*, 2018; Mujubi *et al.*, 2019). Thus, this study was conducted to assess the smallholder dairy cattle population's genetic diversity and population structure in South Africa. Such information is vital for developing a sound breeding programme for the South African smallholder dairy farming system.

2. Materials and methods

Animal sampling and sample collection

All animals were sampled from smallholder dairy farms participating in the National Dairy Animal Recording and Improvement Scheme, which is administered by the Agricultural Research Council (ARC) of South Africa. A total of 196 animals were sampled randomly from herds located in five provinces of South Africa, which were: Eastern Cape (n = 35), Gauteng (n = 40), Free State (n = 38), Kwa-Zulu Natal (n = 44) and North-West (n = 39), in Figure 1.

Hair samples were collected from the animal's tail switch and sealed in labelled envelopes individually to avoid contamination. All samples were taken to the ARC -Biotechnology Platform laboratory in Pretoria, South Africa, for storage and further processing.

Source: United Nations Geospatial. 2007. South Africa. United Nations. Cited 10 October 2023. https://www.un.org/geospatial/file/2270/download?token=J7yUrurN."b

Figure 1: South African map showing the location of herds from which animals were sampled

DNA extraction

The follicles from the root hairs were digested with sodium chloride-tris EDTA, sodium dodecyl sulphate and Proteinase K before DNA isolation. Following the manufacturer's purification protocol, the chemagic™ automated DNA extraction method was used to obtain the purified DNA from lysed follicles (PerkinElmer, chemagen Technology, Midrand, South Africa). The integrity of the genomic DNA was quantified using the Nanodrop (2000) spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Genotyping and quality control

After extraction, the DNA was genotyped using the GeneSeek® Genomic Profiler Bovine Bead Chip consisting of 150 000 SNPs. Genotyping was carried out following the manufacturer's protocol (Neogen, Corporation, Nebraska, USA). Three (3) animals with a call rate less than 90 percent were removed from the analysis. Genotypes of 846 purebred commercial animals, obtained from the Illumina 50K SNP Bovine Chip panel, were made available for the study by the respective breed societies. The breeds represented by these genotypes were selected based on being the founders of smallholder dairy populations and thus, served as the reference populations. These included three specialised exotic dairy breeds: Ayrshire (AYR; n = 200), HOL (n = 222), Jersey (JER; n = 222), and the indigenous Nguni (NGI; n = 202). These genotypes were merged with those of the SHD cattle, and 38 446 autosomal SNPs were in common across the five populations.

Quality control was performed per breed using the PLINK software (Purcell *et al.* 2007). Autosomal SNPs that had a minor allele frequency (MAF) less than 0.05, call rate less than 95 percent and those that deviated from Hardy Weinberg Equilibrium ($p < 0.001$) were removed. The quality controlled population datasets were merged and included 14 926 SNPs. Groups of SNPs with high linkage disequilibrium were pruned to eliminate effects that might cause the ascertainment bias between the populations, as recommended by Kijas *et al.* (2009). Finally, 13 891 SNPs remained after quality control and were used to evaluate the genetic diversity and population structure.

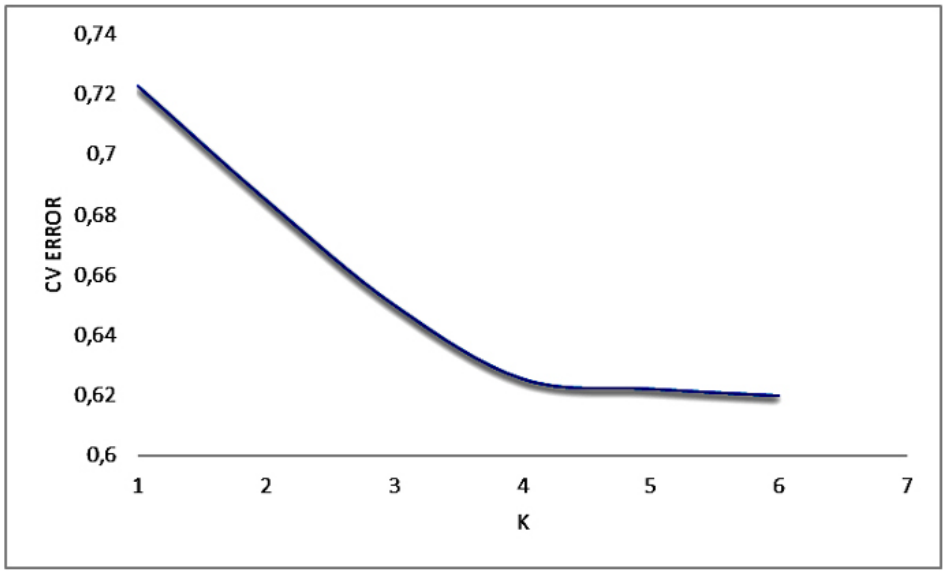
Estimation of genetic diversity and population structure

Allele frequencies were calculated from the SNP genotypes and used to estimate different parameters of genetic diversity, such as the expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (F_{IS}) for each population. Principal Component Analysis (PCA) was performed to investigate breed differentiation, using eigenvalues and eigenvectors constructed from the Genome-wide Complex Trait Analysis (GCTA) software (Yang *et al.*, 2011). The PCA results were visualized using the Genesis package (Buchmann and Hazelhurst, 2015). Population structure was evaluated using the model-based program ADMIXTURE 1.3.0 software (Alexander, Novembre and Lange, 2009) with the number of clusters defined by the low cross-validation (CV) error (K). Estimates of CV error from K = 2 to 6 were plotted to infer the most likely number of clusters. The lowest cross-validation error (0.62) was detected at K = 5, as shown in Figure 2.

3. Results and Discussion

Genetic diversity

Descriptive statistics for the SHD and reference populations are presented in Table 1. All parameters were comparable amongst the populations. Although there was



Source: Authors' own elaboration.

Figure 2: Cross-validation plot for five populations presented in this study (K = 5)

no significant statistical difference in these populations, a slightly increased polymorphism in the SHD and HOL was observed compared to the other three populations. Given the numerous crossbreeding occasions in the SHD population with significant European *Bos taurus* breeds, the population exhibited a comparatively large proportion of SNPs (33 436) with high MAF (0.31). The values of MAF observed across these populations were higher than reported in South African HOL (0.22) and NGI (0.17) (Qwabe, van Marle-Köster and Visser, 2013), Rwandan smallholder dairy (0.29), HOL (0.29) and JER (0.23) (Chagunda *et al.*, 2018) and Indian cattle crossbreds (0.24) (Ahmad *et al.*, 2020).

The average heterozygosity estimates indicated a remarkable proportion of genetic diversity in these populations. Relatively high averages of the expected heterozygosity values (0.40) were observed in SDH, HOL and the AYR populations compared to JER and NGI (H_E = 0.39). The high genetic diversity observed in these populations was higher than the results previously reported by Makina *et al.* (2014) in South African Holstein (0.31) and Nguni (0.28) and other cattle breeds. Heterozygosity values presented in this study were less than those reported in Hanwoo cattle (0.41) (Edea *et al.*, 2013). The observed high genetic variability in SDH offers an opportunity to implement genetic improvement programmes and to prevent adverse effects of the local production environment that are continually changing due to climate change. The average inbreeding coefficient (F_{IS}) was low for all breeds, except in SHD, which corresponds with the low H_O relative to H_E obtained for the SHD population. This was expected because of the prolonged use of the few bulls and artificial insemination predominantly using the random mating practice, which is common in South African smallholder dairy farms.

Population stratification and structure

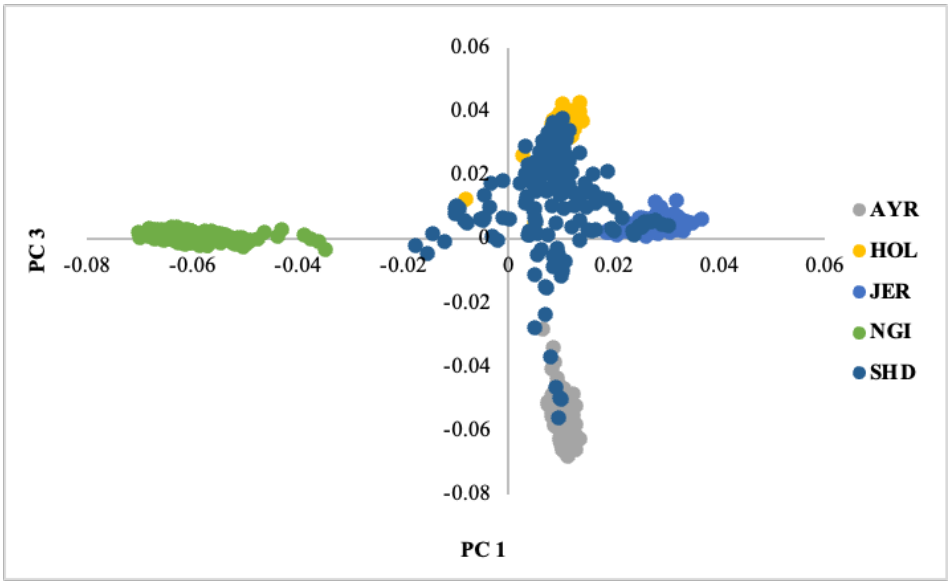
As expected, the PCA plot in Figure 3 formed a homogeneous cluster of the NGI, separated from the specialised dairy breeds (AYR, HOL and JER). On the other hand, the SHD population dispersed widely, although mostfell near to the HOL and to a lesser extent the JER populations, with a few closely traced to the AYR population.

Table 1: Descriptive statistics for the smallholder and reference populations

Population	*N*	Polymorphic loci*	MAF	H_O	H_E	F_{IS}
Smallhoder dairy	189	33 436	0.31 (0.11)	0.39 (0.10)	0.40 (0.09)	0.02 (0.07)
Ayrshire	200	29 020	0.30 (0.12)	0.40 (0.10)	0.40 (0.10)	-0.02 (0.03)
Holstein	222	31 599	0.31 (0.09)	0.40 (0.10)	0.40 (0.09)	-0.01 (0.04)
JerseyER	222	26 118	0.30 (0.12)	0.40 (0.10)	0.39 (0.10)	-0.02 (0.05)
Nguni	202	24 704	0.30 (0.12)	0.39 (0.10)	0.39 (0.10)	-0.004 (0.04)

Source: Authors' own elaboration.

Notes: a * Minor allele frequency (MAF) ≥ 0.05.
b Standard deviations in parentheses, *N* number of animals, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient.

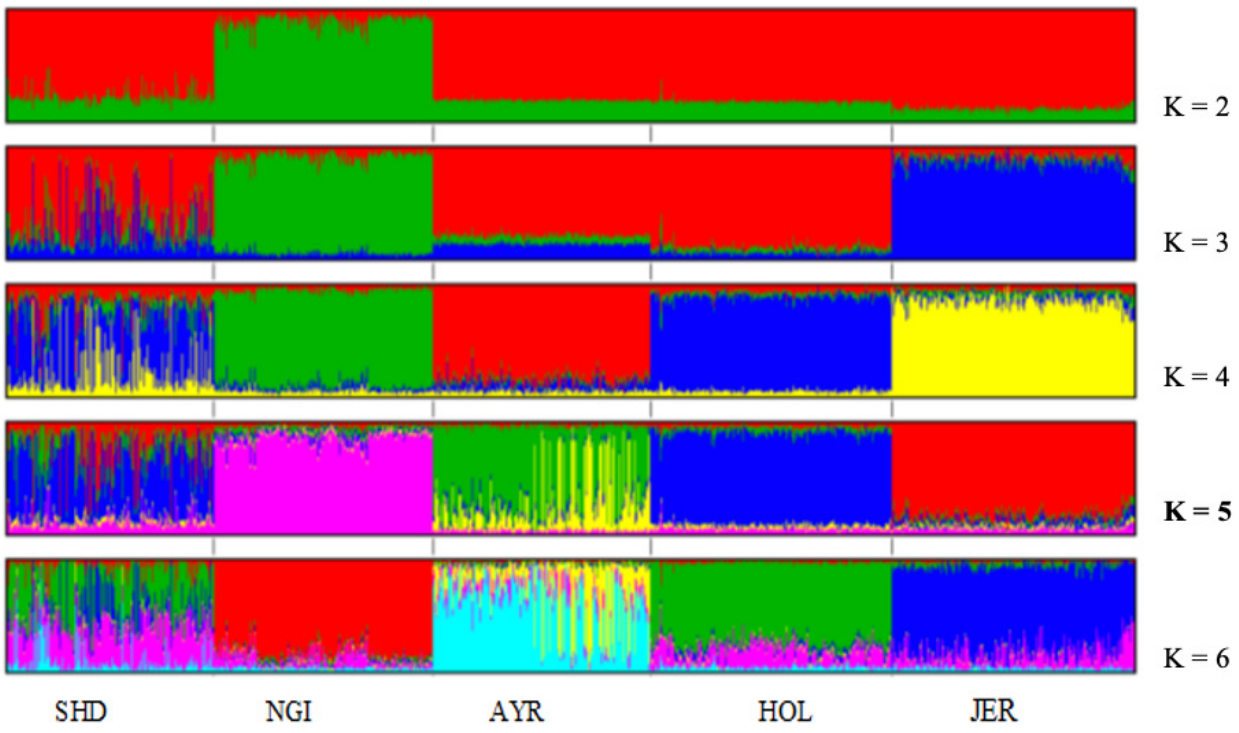


Source: Authors' own elaboration.

Figure 3: Principal component analysis (PCA) plot constructed for PC1 vs PC3 (AYR = Ayrshire, HOL = Holstein, JER = Jersey; NGI = Nguni, SHD = Smallholder Dairy)

An admixture analysis was performed to determine previous crossbreeding events and the existing genetic makeup of the smallholder population, from $K = 2$ to $K = 6$, and the results are shown in Figure 4. At $K = 2$, the indigenous NGI breed formed a distinct cluster. Another cluster represented all the other breeds as one population, pointing out the separation of the exotic cattle from the indigenous NGI breed. The SHD population showed signals of admixture between HOL/AYR and JER at $K = 3$. At $K = 4$, the AYR and HOL became separated, and the SHD presented some gene pool of the AYR, HOL and JER. The results of the admixture analysis at $K = 5$ demonstrated the dominance of HOL and a smaller role of JER in the SHD population, with little AYR, which was consistent with the findings of the PCA. Furthermore, the admixture model indicated signals of unknown genotypes in the AYR population, which requires some investigation.

The PCA and admixture analyses suggest limited use of the indigenous NGI and predominance of the exotic taurine breeds in the crossbreeding on SHD herds. This might be because the herds that were sampled, which participate in the national milk recording scheme, were more developed than the typical smallholder farms. Similar results were reported by Mujibi *et al.* (2019), who observed that SHD herds in Tanzania were largely composed of exotic taurine breeds. Widespread use of indigenous breeds in crossbreeding has, however, been observed in SHD production systems in African countries such as Kenya and Rwanda (Ojango *et al.* 2014; Chagunda *et al.*, 2018). This limited use of indigenous breeds in crossbreeding on South African SHD may compromise cow performance due to the harsh conditions characteristic of this environment. Further sampling is underway to include smaller South African smallholder herds that are known to use the NGI and other indigenous breeds extensively in their crossbreeding. This will make it possible to evaluate a wider range of admixture levels in the smallholder farming environment.



Source: Authors' own elaboration.

Figure 4: Admixture bar plots of breed compositions ($K = 2$ to $K = 6$), with *K* representing the different clusters per breed

5. Conclusions

Smallholder herds sampled in the current study show fairly high levels of genetic diversity and low levels of inbreeding. Crossbreeding between specialised taurine breeds, mostly HOL and JER, is predominantly practised in these herds. Further sampling, to include herds practising crossbreeding with indigenous breeds, will provide a more informative basis for developing a sound breeding programme, based on structured crossbreeding, for the smallholder production system.

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