Epidemiology and molecular determination of benzimidazole resistance in *Haemonchus contortus* (Trichostrongylidae: Nematoda) from naturally infected Merino sheep in Lesotho

by

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science to the Department of Animal Science of the Faculty of Agriculture at the National University of Lesotho

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Dissertation Approval

We, the undersigned approved the work reported herein and that Moeketsi Solomon Phalatsi did it under our supervision in fulfillment of the requirements of the degree of Doctor of Philosophy in Animal Science of the Faculty of Agriculture.

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Declaration

I, Moeketsi Solomon Phalatsi, hereunder signed, declare that this research report is my original work and due references have been provided where other sources were used. I further declare that the work has never been submitted for any degree award in any other university.

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Dedication

To my children Motebang, Lisema, and Sebabatso.

General Abstract

Small ruminants, especially Merino sheep, play an important role in the socio-economic and livelihoods of rural, resource-poor communities in Lesotho. Diseases and poor livestock husbandry practices threaten the small ruminant industry in low-income countries. The present study was designed to investigate the epidemiology, genetic characterization, and detection of anthelmintic resistance in *Haemonchus contortus* in communally grazed Merino sheep in the four ecological zones of Lesotho.

Chapter 1 of the thesis encapsulates the general background and epidemiology of gastrointestinal nematodes (GIN) in small ruminants. Objectives and hypotheses of the study are presented in this chapter. GINs cause substantial economic losses in the small ruminant industry and constitute a limiting factor worldwide. There is, however, little knowledge on the occurrence of GINs, their distribution, intensity, and development of anthelmintic resistance (AR) in small ruminants.

Chapter 2 discusses an overview of *H. contortus* epidemiology in small ruminants. It further delves into GIN species identification, molecular characterization, and molecular detection of anthelmintic resistance.

Chapter 3, presents the anthropogenic barriers and drivers associated with GIN control among smallholder Merino sheep farms in the four ecological zones of Lesotho. A questionnaire survey was designed to assess the activities in livestock management associated with GIN control in four ecological zones (Highlands, Foothills, Lowlands and Senqu River Valley) of Lesotho. Two hundred and twelve (212) respondents were interviewed across the four ecological zones. The study revealed a low literacy rate among Merino sheep farmers across Lesotho with male farmers (77.8%) dominating the industry. Highlands had significantly more animals per farm and predominantly practiced transhumance (93.6%). The respondents used commercial anthelmintic drugs enormously (98%) while herbal and indigenous remedies use was at 57.3%.

Chapter 4, presents a stratified random and cross-sectional study investigating the prevalence, abundance, and geographical distribution of gastrointestinal parasites (GIPs) in Merino sheep. Six hundrend and two (602) Merino sheep from 20 ollection sites were

sampled for faecal matter. The McMaster method of the faecal egg count was used to determine the identity, diversity, and intensity of GIPs from Merino sheep from four ecological zones of Lesotho. Coprocultures were prepared, and third-stage larvae were identified by morphological and morphometric methods under the microscope. The three GIPs identified in the present study comprised, strongyles, *Eimeria* spp., and *Monezia* spp., recording prevalence rates of 64%, 18%, and 1.3%, respectively. The Highlands had the highest strongyle burdens (1170.97 ± 113.134) that were statistically significant ($p < 0.05$) in comparison with other ecological zones. Third-stage larvae from coproculture were subjected to a morphometric species identification protocol and were exclusively identified as *H. contortus*.

Chapter 5, provides a molecular confirmatory test for presumptive morphometric GIN identification owing to the close morphological resemblance between the larvae GINs of ruminants, to give a more conclusive identity. PCR protocol that amplified the Second Internal Transcribed Spacer gene (ITS-2) of the ribosomal DNA was run using universal NC1 and NC2 primer sets. Gel electrophoresis of the ITS-2 resulted in about 350 bp gene segment and the Sanger sequencing protocol confirmed the third-stage larvae identity as 100% *H. contortus*. The present initiative provides the first molecular characterization of *H. contortus* in Lesotho. The phylogenetic tree analysis clustered 17 out of 20 sequences into one clade. The median haplotype analysis produced four haplotypes out of 20 ITS-2 sequences. The mean haplotype and nucleotide diversity values of the ITS-2 gene were 0.7684 ± 0.0689 and 0.00519, respectively. The molecular variation had distribution within topographic populations at 94.35 % while 5.65% was between populations (F_{ST}=0.05651, $p > 0.05$). The ITS-2 gene demonstrated a genetic structure with the most diversity within the population but less differentiation between topographic sub-populations.

Chapter 6, presents the molecular genotyping of the beta-tubulin isotype-1 gene to determine defined point mutations associated with benzimidazole resistance of *H. contortus* in the four ecological zones of Lesotho. Immoderate administration of anthelmintic drugs has been reported to exacerbate the development of anthelmintic resistance (AR) in GINs in small ruminants, adding an unforeseen financial burden to the industry. Genotyping the beta-tubulin isotype-1 partial gene revealed the presence of a benzimidazole resistance-associated mutation (TAC) in codon 200. Three different types of alleles associated with AR in *H.*

contortus were identified: homozygous susceptible (SS = 51.7%), heterozygous (SR = 32.6%), and homozygous resistant (RR = 15.7%). The present study presents the first molecular evidence of AR of *H. contortus* from Merino sheep in Lesotho. The phylogenetic tree analysis of the beta-tubulin isotype-1 partial gene clustered all sequences into one clade, depicting a close evolutionary relationship among all seven sequences. The partial gene exhibited haplotype and nucleotide diversity of 1.000 ± 0.076 and 0.01891, respectively. The result depicted absolute haplotypic differentiation among all seven sequences.

Chapter 7, entails general discussions on the interconnections of different factors from the respective project's sub-sections. The overwhelming intensity and vast distribution of *H. contortus* in the study suggest it is the predominant cause of helminthiasis in Merino sheep in Lesotho. The present study suggests that extensive use of anthelmintic drugs in Merino sheep is a probable cause of AR development in *H. contortus*. Genetic characterization is the basis for molecular epidemiology and molecular tracer studies that inform on prevention initiatives and resistance detection. Timely and periodic parasite surveys on AR are essential for strategic management, prevention, and control of GINs.

Keywords: Gastrointestinal nematodes, *Haemonchus contortus*, Merino sheep, Anthelmintic resistance, ITS-2, Haplotype diversity, Nucleotide diversity, genetic differentiation, Single nucleotide polymorphism, Beta-tubulin isotype-1

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List of Abbreviations and Nomenclatures

AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
AR	Anthelmintic resistance
AS-PCR	Allele-specific PCR
DNA	Deoxyribonucleic Acid
EPG	Eggs per gram of faeces
FAMACHA	FAfa MAlan CHArt
FEC	Faecal egg counts
FECRT	Faecal egg count reduction test
GDP	Gross Domestic Product
GIN	Gastrointestinal nematode
GIP	Gastrointestinal parasite
Hd	Haplotype diversity
ITS-2	Second Internal Transcribed Spacer gene
LAMP	Loop-Mediated Isothermal Amplification
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
OPG	Oocysts per gram
P167	Amino acids position at codon 167
P198	Amino acids position at codon 198
P200	Amino acids position at codon 200
PCR	Polymerase chain reaction
Phy	Phynelalanine
Pi (π)	Nucleotide diversity
rDNA	Ribosomal DNA
SNP	Single nucleotide polymorphism
SRV	Senqu River Valley
STE	Sheath tail extension

•	Tyr	Tyrosine
•	WAMPP	Wool and Mohair Promotion Project
•	WAAVP	World Association for the Advancement of Veterinary Parasitology

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Appendices

Appendix A: Nucleotide sequence polymorphism of 231 bp of a complete segment of ITS-2 of the ribosomal DNA *Haemonchus contortus* showing nucleotide variations between Lesotho isolates using *H. contortus* accession number X78003

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Appendix J: Published article from the present thesis.

CHAPTER 1

General Introduction

1.1. Background

1.1.1 Socioeconomics of sheep rearing in Lesotho

Small ruminant rearing constitutes a major socio-economic activity for rural populations in Lesotho (Hunter, 1987). Wool and mohair sales have dominated Lesotho's livestock production gross domestic product (GDP) for several decades (Hunter, 1987; Mafisa, 1993; Hoag, 2018). With Merino sheep reared as the dominant species, Lesotho exported wool fleece with market sales of about $33 million in 2017/2018 (BKB Fibretrack, 2018) to $38 million in 2021 (Trading Economics, 2021). However, recent wool production is suspected to be below optimum due to setbacks associated with animal morbidity, mortality, and poor husbandry practices (WAMPP, 2021). Research on gastrointestinal parasites (GIPs) in Southern Africa has highlighted two major groups of parasites infecting small ruminants: helminths (nematodes, cestodes, and flukes) and protozoa (coccidia) (Mpofu *et al.*, 2020; Kusiluka and Kambarage, 1996). Taylor (2010) suggested that small ruminants are at risk of endoparasites and ectoparasites that have a serious impact on the livestock economy. Gastrointestinal nematodes (GIN) are among the most economical and widely distributed, especially for grazing livestock (Trading Economics, 2021). Moreover, parasitic infections, inadequate nutrition, and substandard husbandry practices threaten the successful small ruminant industry worldwide (Dey *et al.*, 2019). Major retrogressive results in livestock production include low animal weight, low wool tonnage, poor wool quality, and animal death (Papadopoulos *et al.*, 2003; Besier *et al.*, 2016; Emery *et al.*, 2016; Patel *et al.*, 2019). The cost of GIN control programs and management of anthelmintic resistance (AR) development also escalates losses to the industry (Iqbal *et al.*, 2005; Munguía-Xóchihua *et al.*, 2018).

The severity, transmission, and progression of GINs in small ruminants are dependent on factors such as animal movements, microenvironments, infection rates, host immunity levels, animal host age, and nutritional status (Roeber *et al.*, 2013; Naeem *et al.*, 2021). Livestock management and movements, either for socio-economic purposes or better pastures in resource-poor communities, affect parasites' spatial and topographic distribution (Besier *et al.*, 2016). Socio-economic activities such as trade and pasture seeking may contribute to gene flow within and between parasite populations (Emery *et al.*, 2016; Kandil *et al.*, 2018). Invariantly, seasonal variations affect the occurrence and abundance of free-living larval stages on the pastures as a key indicator of relative risk and the potential for reinfections within the host population (Iqbal *et al.*, 2005).

1.1.2 Haemonchus contortus infections in Small Ruminants

Haemonchus contortus (the barber's pole worm) is a parasitic roundworm belonging to phylum Nematoda and parasitizes a wide range of animals including wild and domesticated ruminants (Shen *et al.*, 2017; Almeida *et al.*, 2018; Dey *et al.*, 2019). The parasite is ingested by the host while grazing and resides in the abomasum where it feeds on the host's blood (Besier *et al.*, 2016). The parasite causes anaemic outbreaks characteristic of haemonchosis, a disease responsible for substantial morbidity and mortality in small ruminants around the globe (Papadopoulos *et al.*, 2003; Troell *et al.*, 2006; Jabbar *et al.*, 2006; Matebesi-Ranthimo *et al.*, 2014). *Haemonchus contortus* is regarded as the most pathogenic GIN of ruminants, endemic in tropical and subtropical areas, especially the resource-poor livestock farming communities (Lichtenfels *et al.*, 1994; Troell *et al.*, 2006; Murad *et al.*, 2018; Selemon, 2018; Wang *et al.*, 2018; Dey *et al.*, 2019). Animals with acute infection show pale mucous membranes, thriftiness, emaciation, bottle jaw edema and eventually die (Besier *et al.*, 2016). Consequently, *H. contortus* is a limiting factor in the small ruminant industry (Troell *et al.*, 2006; Mphahlele, 2021). The nematode exhibits a characteristic trichostrongylid life cycle comprising free-living larvae on pastures, parasitic development stages and adults in the abomasum of the host, where a female is highly fecund laying about 10 000 eggs daily, which are shed into the environment together with the host's faeces (Fernández-Ruvalcaba *et al.*, 1994). The interaction between factors such as environmental conditions, parasite intensity, distribution, larval pasture contamination, and anthropogenic activities constitute

the epidemiological context of *H. contortus* (Bolajoko and Morgan, 2012; Molento *et al.*, 2016).

1.1.3 Diagnosis of gastrointestinal parasites

Determination of disease trends and conceptualization of their daunting epidemiological effects begins with disease diagnoses and parasitic identifications. The importance of correct disease diagnosis cannot be overemphasized as it provides valuable information for identifying disease agents for the decisive development of strategies for prevention, management, and control (Molento *et al.*, 2016). For several decades, microscopy has been central to laboratory diagnostics, especially in identifying pathogenic nematodes by screening eggs from faecal material and providing data for a general viewpoint without necessarily determining species identity (van Wyk and Mayhew, 2013). Notwithstanding its extensive use, several scholars reported that comparative morphological identification of GIN stages to genus and species level was relatively cumbersome, even though it still provided beneficial results in laboratory diagnostics (Heise *et al.*, 1999; van Wyk and Mayhew, 2013; Zarlenga *et al.*, 2016).

The close morphological similarity between *H. placei* in cattle and *H. contortus* in sheep poses a challenge to the timely and precise distinctions between the two species, especially in sympatric populations (Zarlenga *et al.*, 2016). Parasitological studies conducted by microscopy revealed nematodes, coccidia, and tapeworms were three major GIPs infecting Merino sheep and Agora goats in Lesotho (Moiloa *et al.*, 2020; Matsepe *et al.*, 2021; Mahleha *et al.*, 2021; Phalatsi *et al.*, 2022). Relatively more sensitive and accurate protocols such as molecular methods have been instrumental in distinguishing between the two close *Haemonchus* species through genotyping (Stevenson *et al.*, 1995; Gilleard *et al.*, 2006). Matsepe *et al.* (2021) reported 100% *H. contortus* from the morphological identification of third-stage larvae in goats in four ecological zones of Lesotho. The initial reports by Moiloa *et al.* (2020); Mahleha *et al.*, (2021) Matsepe *et al.* (2021), and Phalatsi *et al.* (2022) portrayed GINs as the most abundant and prevalent, hence need for further investigation and appropriate response to lessen the clinical and economic impact in Lesotho.

1.1.4 Molecular epidemiology and anthelmintic resistance development

Molecular and genetic protocols have been widely adopted in parasite identification as they have proven effective, timely, and accurate (Emery *et al.*, 2016). These techniques utilise genetic markers to accurately identify all life stages, from genus to sub-species level, in one polymerase chain reaction (PCR) (Knoll *et al.*, 2021). Laboratory investigations use molecular and morphological methods concurrently, depending on the diagnostic and epidemiological objectives of the assignment (Stevenson *et al.*, 1995; Mangkit *et al.*, 2014). Furthermore, molecular analysis was used in parasite population studies and genetic tracer studies. Emery *et al.* (2016) expressed ways molecular work is used in determining phenotypic traits in species and how they translate into genotypes and vice versa.

Surveys conducted in Southern Africa and Lesotho predominantly focused on parasite prevalence, intensity, and control measures employed in small ruminants (Mphahlele *et al.*, 2021; Moiloa *et al.*, 2020; Mahleha *et al.*, 2021; Phalatsi *et al.*, 2022). There was a long history of extensive use of anthelmintic drugs against gastrointestinal parasites in Merino sheep and Angora goats farms in Lesotho (Mahlehla *et al.*, 2021; Moiloa *et al.*, 2021). Lesotho sheep farmers use anthelmintic drugs for GIN control which are commonly administered include, benzimidazole, niclosamide, and ivermectin, (Moiloa *et al.*, 2020; Mahlehla *et al.*, 2021). The increased intensity and long-term use of anthelmintic drugs have been reported to result in the AR development of GIN populations in farming communities worldwide (Gilleard *et al.*, 2006). Benzimidazole has been reported as the major circulating drug of choice among farmers, and there are suspicions of GIN treatment failure and the development of AR (Zhang *et al.*, 2016).

Epidemiology and resistance development to benzimidazole drugs GINs have not been adequately conceptualized among Merino sheep farming communities in Lesotho. The present study used morphometrical and molecular methods to identify GINs infecting sheep at genus and species levels. Consequently, intra- and inter-specific genetic diversity and population structure of *H. contortus* in Lesotho was determined. Phylogenetic relationships among Lesotho isolates and the occurrence of genotype inferences need to be established and comparisons instigated against *H. contortus* sequences from Genbank for a closer examination. Determining the occurrence and distribution of AR-associated mutations and the frequency of alleles responsible for resistance among *H. contortus* populations can

provide valuable information for stakeholders to develop control strategies based on an informed background. Consequently, the complexity and interrelations of different factors in the management of ruminants make it prudent to investigate the interaction between anthropogenic effects, animal movement, AR development status, *H. contortus* molecular diversity, and gene flow for better conceptualization of the GIN epidemiological outlook in Lesotho.

1.2. The Problem Statement

Wool and mohair are major livestock export commodities, constituting about 58% of all agricultural exports (WAMPP, 2021). The small ruminant industry in poor-resourced communities depends on communal grazing, and a low-cost provision of nutritional requirements for farm animals (Bekuma and Dufera., 2019). Different topographical strata are hypothesized to have diverse ecological regimes for farmers and their flocks, necessitating area-specific rangeland management, drug administration, and overall husbandry. Range management, entrenched in the land tenure system of Lesotho, involves pasture rotation with transhumance, which is practiced extensively as a method of seasonal and topographic pasture rotation (Mbata, 2001). The occurrence, intensity, and sustenance of infections within the host populations are mainly influenced by the interaction between the host, the disease agent, and the environment (Acevedo-Whitehouse and Duffus, 2009). The complex interrelationships between the abovementioned factors and anthropogenic activities exemplify the epidemiological context of haemonchosis in Lesotho. With decades of extensive and haphazard use of anthelmintic drugs in Lesotho, the development of AR for different drug groups is highly possible, and even worse, multidrug resistance. A compounding effect of overstocking and a mix of various farm animals on the same communal pastures may perpetuate the continuous risk for parasite infections. Previous research has also shown that GIPs are common infections in Merino sheep and Angora goats in all ecological zones (Moiloa *et al.*, 2020; Matsepe *et al.*, 2021; Mahleha *et al.*, 2021). Reinfections by *H. contortus* and AR development tend to perpetuate morbidity among ruminant populations (Molento *et al.*, 2016). The cost associated with GIN control and AR management increases the livestock industry's economic expenditure (Iqbal *et al.*, 2005; Munguía-Xóchihua *et al.*, 2018). It is hypothesized that the current state of GIN reinfection will escalate unabated if no countermeasures are taken to improve the status quo. Ruminant

GIN infections, and more especially haemonchosis, may consequently exacerbate economic losses, causing low wool yield per animal, increased morbidity, and animal mortality if no strategic interventions are introduced. Epidemiology aims to evaluate the clinical, anthropogenic and ecological factors that influence the survival and transmission of disease-causing pathogens to manage and control diseases and avert economic losses and risks to human health (Besier *et al.,* 2016). The present research initiative was set up to bridge the knowledge gap and provide valuable epidemiological information for a better understanding of haemonchosis, GIN control, and AR incidences in Lesotho.

1.3. Study Objectives

1.3.1. General objective

To investigate the epidemiology and molecular characterisation of benzimidazole resistance in *H. contortus* affecting Merino sheep across the four ecological zones of Lesotho.

1.3.2 Specific Objectives

- To determine the Merino sheep farmers' perspectives on factors that influence gastrointestinal nematodes' management, control, and anthelmintic resistance development across the four ecological zones of Lesotho.
- To evaluate the prevalence, distribution, and intensity of gastrointestinal parasites from Merino sheep in Lesotho.
- To characterize *H. contortus* third-stage larvae by morphometric and molecular procedures.
- To determine the phylogenetic status, genetic diversity, and population structure of *H. contortus* in Lesotho.
- To evaluate the occurrence, distribution, and frequency of alleles associated with benzimidazole resistance in *H. contortus* in Lesotho.

1.3.3 Study Hypotheses

- Null hypothesis (H_o): There are no significant differences in factors influencing GINs management and control of GINs among Merino sheep farms in the four ecological zones of Lesotho.

- Alternative Hypothesis (H_1): There are significant differences in factors influencing the management and control among Merino sheep farms in four ecological zones of Lesotho
- Null hypothesis (H_o): There are no significant differences between the diversity and intensities of gastrointestinal parasites in Merino sheep in four ecological zones of Lesotho.
- Alternative Hypothesis (H_1): There are significant differences between the diversity and intensities of gastrointestinal parasites in Merino sheep in four ecological zones in Lesotho.
- Null hypothesis (H_o): No significant genetic variation exists between *H. contortus* sub-populations across the four ecological zones in Lesotho.
- Alternative Hypothesis (H_1): There is a significant genetic variation between *H. contortus* sub-populations in four ecological zones in Lesotho.
- Null hypothesis (H_o): No benzimidazole resistance-associated alleles exist among ecological zones's sub-populations of *H. contortus* in Lesotho.
- Alternative Hypothesis (H_1): Benzimidazole resistance-associated alleles exist among the ecological zones's sub-populations of *H. contortus* in Lesotho.

1.4 Significance of the study

The present study addresses aspects of *H. contortus* epidemiology in Merino sheep across four ecological zones of Lesotho. As a result of its high pathogenicity, *haemonchosis* was suspected to be the limiting factor in small ruminant production, resulting in serious economic losses. Merino sheep production is enshrined in the Lesotho National Strategic Development Plan II Strategic Focus, 2023/24–2027/28. Consequently, the National Livestock Development Policy recognises the National University of Lesotho (NUL) as a stakeholder institution responsible for research and providing technical solutions for relevant and timely livestock production developments.

The present study's findings will be used to ascertain the diversity, prevalence, distribution, and abundance of GIPs of Merino sheep across the four ecological zones of Lesotho. The information will be valuable to conceptualise the epidemiological dynamics of GIPs, with special emphasis on *H. contortus*. The study will assist in identifying major management

bottlenecks and hotspots for AR occurrence and intensity. The information is envisaged to form part of the knowledge base for local government authorities, farmers, livestock extension service personnel, and other stakeholders in the overall livestock management and production. Furthermore, the information from the study will enhance policy development on livestock production, GIN prevention, livestock movement, range management, drug use, and control of AR development. The economics of Merino sheep in Lesotho become the background for priority and research interest in AR.

CHAPTER 2

2. Literature review

2.1. Overview of the economy of sheep production in Lesotho

Lesotho is a small country landlocked in South Africa and characterized by four main ecological zones, namely, The Highlands, Foothills, Lowlands, and Senqu River Valley. Small ruminant production in Lesotho is typical of traditional pastoral farming, which forms the backbone of livelihoods and exemplifies major economic activities for Lesotho rural communities (Hunter, 1987; Turner, 2013; Mokhethi *et al.*, 2015; Nhemachena *et al.*, 2016). In 2015, the livestock sub-sector contributed about 60% of Lesotho's agricultural GDP (FAO, 2015). Small ruminants are a significant source of food and nutrition for millions of low-income rural populations in different parts of the globe (Simões *et al.*, 2021; Oluwatayo and Oluwatayo, 2012). The Merino sheep and Angora goats are multi-purpose animals kept mainly as wool and mohair producers. The country's cool climate and mountainous terrain provide ideal conditions for the rearing of sheep and goats (Obioha., 2010; Turner., 2013). The small ruminant industry provides employment opportunities, income for farmers, and livelihoods for rural communities (Mokhethi *et al.*, 2015). Lesotho exports its wool and mohair to various countries, including China, which has been a significant buyer of these fibers (Trading Economics, 2021).

The diversity of livestock farming systems is mainly determined among other factors, by (1) the level of intensification in utilising food resources, (2) the animal species and breeds involved, (3) the production system, (4) the area of cultural roles, and (5) the farmer's socio-economic dynamics (Bernues *et al.*, 2011). Small and subsistence farmers characterize Lesotho's small ruminant industry which existed for over a century (Hunter, 1987). Sheep in low-income communities are grazed in communal rangelands. Sheep farms comprise a mix of different farm animals such as goats, cattle, horses, and donkeys (Bekuma and Dufwera, 2019). Communal rangelands in resource-scarce and extensive farming communities are an unescapable commodity that provides daily animal nutritional requirements. (Turner, 2013; Kandil *et al.*, 2018; Bekuma Dufera, 2019). Communal grazing is a major risk for livestock as it continuously exposes animals to GIP infections in

contaminated rangelands (Kandil *et al.*, 2018). Figure 2.1 shows a headman leading a mixed flock of sheep and goats into the communal pasture in the highlands of Lesotho.

Figure 2.1: A head boy leading sheep and goats in the mountainous Highlands of Lesotho in Mokotane village near Mants'onyane.

Lesotho's geographical proximity to South Africa enables ease of livestock trades and wild animal movements, and there have been cross-border livestock theft activities between these two countries, all presumable sources of veterinary infection transmissions (Dzimba and Matooane, 2005). Farmers in Lesotho have been enhancing their flock by purchasing Merino and Angora breeds to improve the quality and quantity of their wool and mohair production. Consequently, there is a potential risk of disease transmission between the two countries. On the other hand, Lesotho has over a century engaged in small ruminant agribusiness but GIN research only dates to the recent decade. The trichostrongyle *H. contortus* is one of the most pathogenic and widespread GIN of small ruminants worldwide (Ayana and Ifa, 2015; Besier *et al.*, 2016; Matsepe *et al.*, 2021). For the sustainability of GIP control in ruminants, there is a need for a multidisciplinary approach to integrate anthelmintic drug administration, grazing and pasture management, biological control, vaccine development, administration,

and policy formulation. Furthermore, genetically resistant hosts and strategic GIN management and control of AR development are used against the parasite scourge (Bukhari and Sanyal, 2011).

2.2. Gastrointestinal parasite diversity and prevalence of *Haemonchus contortus* in small ruminants in Lesotho

Gastrointestinal infection is one of the limiting factors to successful small ruminant production worldwide (Asif *et al.*, 2008; Cai and Bai, 2009; Singh *et al.*, 2013). Nematodes, cestodes, protozoa, and tissue flukes are the four main GIP groups that infect ruminants in Southern Africa, including Lesotho (Tsotetsi and Mbati, 2003; Raza *et al.*, 2007; Mphahlele *et al.*, 2021; Moiloa *et al.*, 2019; Matsepe *et al.*, 2021; Mahlehla *et al.*, 2021). Gastrointestinal parasitess occupy different predilection sites within the ruminant gastrointestinal tract. Common GINs that infect small ruminants across the globe include *H. contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Cooperia curticei*, and *Oesophagostomum* spp., *Nematodirus* spp., *Trichuris ovis*, *Bunostomum trigonocephalum*, and *Strongyloides papillosus* were less prevalent (Soulsby, 1982; Raza *et al.*, 2007). *Haemonchus contortus*, *Teladorsagia/Trichostrongylus, Oesophagostomum columbianum, Calicophoron,* and *Fasciola* were reported in different areas of South Africa (Tsotetsi and Mbati., 2003; Mphahlele *et al.*, 2021). *Haemonchus contortus* was recorded as the most abundant and prevalent GIN species in the Eastern Cape Province of South Africa (Gwaze *et al.*, 2009).

The research initiatives undertaken in Lesotho mainly comprised farmers' perceptions of parasites and their control, parasite prevalence and abundance in small ruminants, horses, and donkeys (Moiloa *et al.*, 2019; Moiloa *et al.*, 2020; Mahlehla *et al.*, 2021; Kompi *et al.*, 2021; Matsepe *et al.*, 2021). Gastrointestinal nematodes prevalence rates of 53.9%, 67.9%, and 48.4% were recorded by Mahlehla *et al.* (2021), Phalatsi *et al.* (2022), and Phororo *et al.* (2023), respectively, among Merino sheep from different areas in Lesotho. In these studies, there were notable variations in prevalence and abundance among different ecological zones. Different livestock species grazed in common pastures may perpetuate cross-infection among them. Matsepe *et al.* (2021) recorded the overall prevalence of

strongyles in goats as 64.7% in Lesotho, while Moiloa *et al.* (2019) recorded 79% and 74% in Quthing and Maseru districts, respectively.

Notwithstanding, the variations from different surveys, strongyles were generally the most abundant group in prevalence and parasite burdens (Moiloa *et al.*, 2019; Moiloa *et al.*, 2020; Matsepe *et al.*, 2021; Mahlehla *et al.*, 2021; Phalatsi *et al.*, 2022). Lesotho studies on GIP revealed a positive relationship between prevalence and parasite abundance. Nevertheless, preliminary studies could not characterize GIP at the species level, but only gave the phylum. For example, the diagnosis based on the faecal egg flotation protocols, using the McMaster technique, revealed three main GIP groups: nematodes, cestodes, and coccidians (Moiloa *et al.*, 2019; Matsepe *et al.*, 2021; Mahlehla *et al.*, 2021; Kompi *et al.*, 2021). Matsepe *et al.* (2021) reported on the morphological identity of nematode third-stage larvae in goats, in which they reported all strongyles identified as 100% *H. contortus*. Figure 2.2 shows the seasonal burdens of GIPs with corresponding monthly rainfall and temperatures in the Maseru district, Lesotho. The graphical presentation also shows peak infections during the summer months, during which there was relatively high precipitation and temperature (Phalatsi *et al.*, 2022).

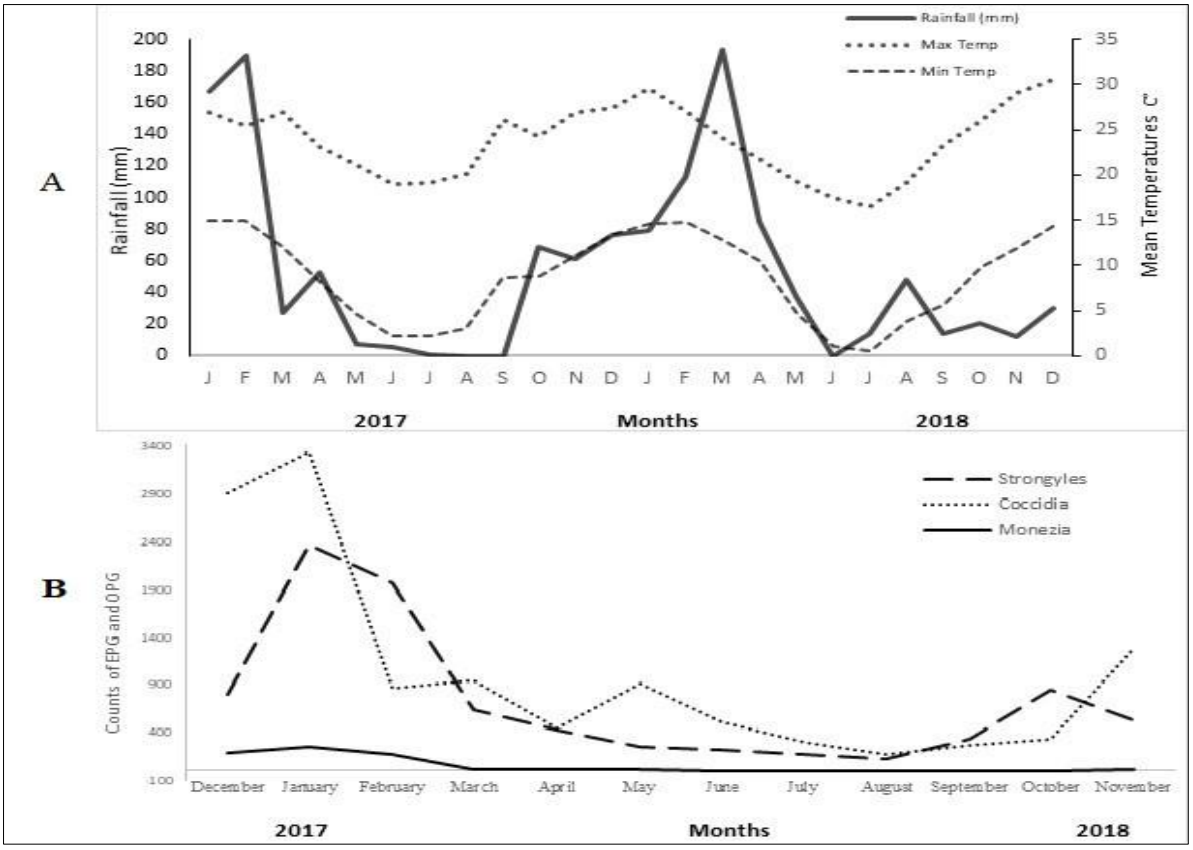

Figure 2.2: A, Lesotho indicative monthly rainfall (mm) and temperatures (℃), B. Monthly mean eggs per gram of faeces (EPG) counts for helminths (Strongyles and *Monezia*) and oocysts per gram (OPG) of *Eimeria* spp from sheep in Maseru District during 2017-2018 (Phalatsi *et al*., 2022).

2.3. Etiology and life cycle of *Haemonchus contortus*

The GIN, *H. contortus* causes a disease called haemonchosis, which is globally common and economically worrisome in small ruminants' production (Lichtenfels *et al.*, 1994; Dey *et al.*, 2019). *Haemonchus contortus* was first described by Karl in 1803 as a member of the family Trychostrongilidae, mainly identified by their characteristic bursar in males; hence the name "bursates". The female *Haemonchus* is distinguished by its characteristics intertwining of the red blood-filled intestine and white elongated reproductive organs, forming a "barbers' pole" like outlook. The feature gave rise to its common name "barber's pole worm." Moreover, the presence of a vulva flap in females serves as a key diagnostic feature. Alternatively, male *Haemonchus contortus* are identified by their distinctive barbed-tipped spicules. Twelve species make up the genus *Haemonchus*, namely *H. contortus, H. placei,*

H. similis, H. longistepes, H. bedfordi, H. mitchelli, H. vegliali, H. lawrencei, H. okapiae, H. krugeri, H. horaki, and H. dinnik (Angulo-Cubillán *et al.*, 2007). *Haemonchus contortus* and *H. placei* are the closest relatives of the genus. Though the classification of nematodes is still a subject for debate among different scholars, a method used to classify *H. contortus* is represented as follows:

- *Kingdom:* Animalia
- Phylum: Nematoda
- Class: Chromadorea
- Order: Strongylida
- Superfamily: Trichostrongyloidae
- Family: Trichostrongylidae
- Genus: *Haemonchus*
- Species: *Haemonchus contortus*

H. contortus infects a wide range of wild and domesticated ruminants, including sheep (Almeida *et al.*, 2018). The disease is most prevalent in tropical and subtropical regions worldwide and is responsible for major helminthiasis outbreaks in small ruminants (Troell *et al.*, 2006; Angulo-Cubillán *et al.*, 2007; Wang *et al.*, 2018; Murad *et al.*, 2018; Dey *et al.*, 2019). Infective larvae (L_3) from pastures are ingested by a host and reside in their predilection site, the abomasum of ruminants. In the host, they perforate the gastric mucosa to suck blood, causing blood loss (Lichtenfels *et al.*, 1994).

Trichostrongylids' life cycle comprises the host-dwelling phase (exsheathed L_3, forth-stage larva-L_4 and adult worms) and off-host phases (eggs and larval instars) in the external environment (Zarlenga *et al.*, 2016; Besier *et al.*, 2016; Naeem *et al.*, 2021). The trichostrongylid nematodes, such as *Haemonchus* spp., exhibit a direct type of life cycle. The adult nematodes lodge in the abomasum, feed on the host's blood, and mate (Le Jambre, 1995; Besier *et al.*, 2016). The females subsequently lay eggs, which are then passed in faeces to the outside environment during defecation (Molento *et al.*, 2016). Under favourable conditions, eggs in the faecal matter develop into the first-stage larvae (L_1) still inside an egg. First-stage larvae subsequently molt into second-stage larvae (L_2), which hatch and feed on microorganisms in the faecal matter and molt into third-stage larvae (L_3) (Molento *et al.*,

2016) (Figure 2.3). The ensheathed L_3 becomes active and undergoes host seeking by moving from the faeces and questing for a vantage position on the vegetation, ready for uptake by a potential host (Roeber *et al.*, 2013). The L_3s are then ingested with the vegetation while the host animal feeds and molts into L_4s, which then inhabit the abomasum, exsheathment occurs at the L_3 stage and in the rumen, and further develops into adult worms. *Haemonchus contortus* exhibits high fecundity levels, laying about 5000–10,000 eggs daily, and also exhibit high genetic variation (Besier *et al.*, 2016).

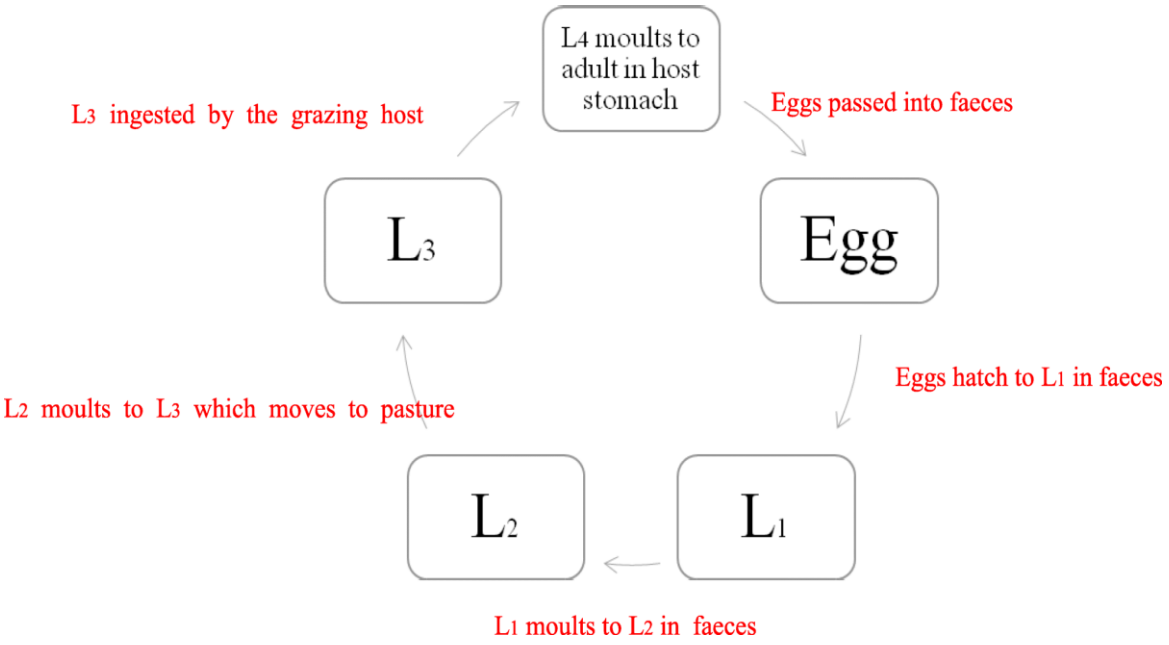

Figure 2.3: A generalized schematic representation of the lifecycle of *H. contortus* of small ruminants. First-, second-, and third-stage larvae (L_1, L_2, and L_3, respectively) are found in the external environment. The exsheath L3 are found in the host and the fourth-stage larva (L_4) in the predilection site in the small ruminant abomasum. (Naeem, *et al.*, 2021).

2.4. Epidemiology of *Haemonchus contortus*

Grazing livestock is exposed to a constant risk of infection by a plethora of parasites and infectious diseases, especially in farming communities that inevitably utilise communal pastures (Bisset *et al.*, 2014; Besier *et al.*, 2016). The epidemiology of sheep *H. contortus* can be explained through the concept of the epidemiological triad (Figure 2.4). The triad conceptualizes the ‘parasite's intrinsic and extrinsic factors (the environment), predisposing the host to the infection (Acevedo-Whitehouse and Duffus, 2009). The intensity of a disease

depends on complex interactions of factors between the parasite, the host, and the environmental conditions that influence host's susceptibility to the pathogen (Bolajoko and Morgan, 2021). Other host-borne factors affecting *H. contortus* epidemiological status are age, population density, sex, genetic variation, and nutritional and immunological status. (Naeem *et al.*, 2021). Moreover, anthropogenic factors such as parasite control regimes, socioeconomic activities, and range management strategies play a vital role in the epidemiological dynamics of parasites (Besier *et al.*, 2016; Molento *et al.*, 2016). Factors influencing epidemiology dynamics include seasonal weather conditions, pasture microhabitats, host movement, and parasite genetic diversity (Besier *et al.*, 2016). O'Connor *et al.* (2007) reported that relative humidity and faecal moisture content were responsible for the viability of nematode eggs, while temperature determined the length of the developmental cycle. Fernández-Ruvalcaba *et al.* (1994) performed experiments on larval survival during different seasons and, based on the results, concluded that humidity was a major limiting factor for the off-host development and survival of *H. contortus*. The seasonal variations and abundance of free-living larval stages on the pastures also contribute significantly to the index of relative risk and prospective infections within the host population (Iqbal *et al.*, 2005; Cerruti *et al.*, 2010). The survival and rate of development of free-living stages (L_1 to L_3) of GINs depend largely on physical environmental conditions (O'Connor, 2006; Bolajoko and Morgan, 2012; Besier *et al.*, 2016; Molento *et al.*, 2016). The control of *H. contortus* targets parasitic and free-living stages by interrupting their reproduction, survival, and progression into subsequent stages. The protocols lessen active parasite numbers and their chances of finding a potential host for sustaining their reproduction. Invariably, the successful management of parasite scourges significantly reduces imminent economic losses.

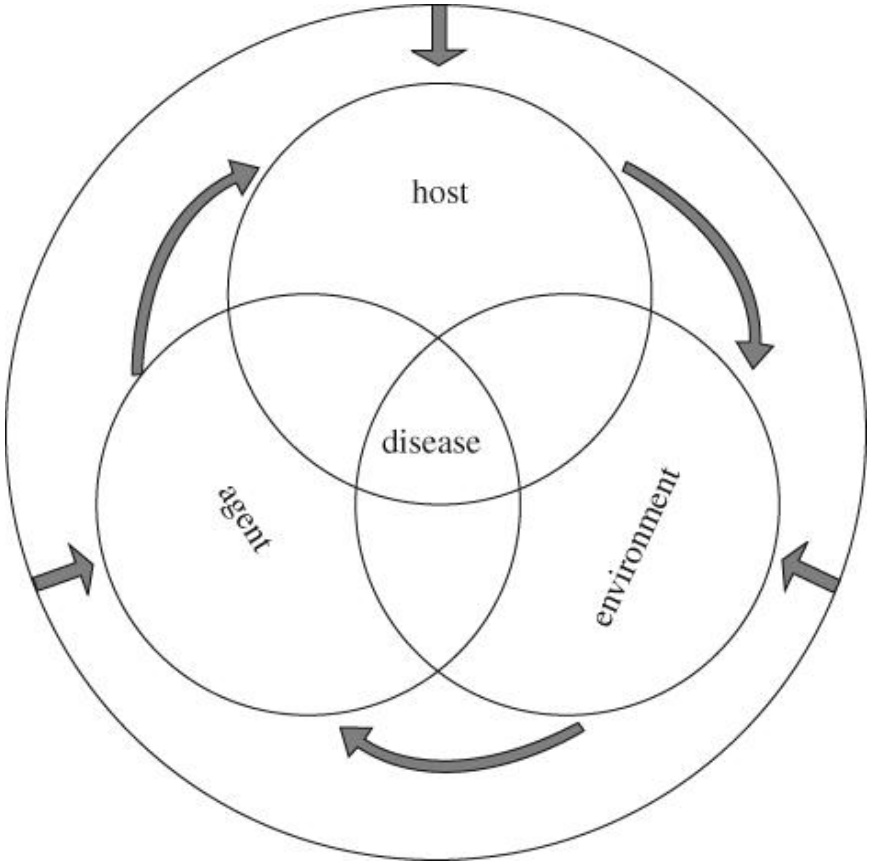

Figure 2.4: Epidemiological triad representing the relationships between ruminants (host), *H. contortus* (disease agent), and the 'environment' in disease etiology (adapted from Acevedo-Whitehouse and Duffus, 2009).

2.5. Molecular epidemiology

The molecular epidemiology incorporates molecular biology in studying disease dynamics, such as genetic diversity, population genetic structure, and gene flow between parasite populations (Blouin *et al.*, 1995; Akkari *et al.*, 2013; Kandil *et al.*, 2018; Pitaksakulrat *et al.*, 2021). Molecular techniques are developed as diagnostic tools for detecting GIN infections (Kandil *et al.*, 2018). PCR-based methods target specific genetic markers for rapid and accurate parasite detection and characterization (Chaudhry *et al*., 2016). The protocols further provide insights into the parasite's evolution, migration patterns, potential sources of infection, and determination of AR among GINs (Chaudhry *et al.*, 2016). Molecular determination of AR is applied by identifying genetic markers associated with resistance to assess the occurrence and intensity of resistant alleles and to conceptualize mechanisms of resistance development (Önder *et al.*, 2016). Consequently, assessing the transmission

patterns of *H. contortus* is crucial for the broader conceptualization of this daunting problem and becomes a key step in the design of parasite management and control strategies. Molecular tools can therefore be used to trace the origin of infections, study the movement of parasites between hosts and geographic regions, and assess the role of different hosts in the transmission cycle (Blouin *et al.*, 1995). In a vast field of veterinary science, adventuring in host-parasite interactions also increases the knowledge base in understanding how host immune response, parasite virulence factors, and mechanisms of immune evasion interact. Molecular techniques such as DNA sequencing and phylogenetic analysis are crucial in the continuous reconstruction of the evolutionary history of *H. contortus* and its related species (Blouin *et al.*, 1995). Consequently, providing insights into its origins, diversification, and adaptation to different host environments (De Salle *et al.*, 2017; Blouin *et al.*, 1995). Vaccine development against *H. contortus* also requires knowledge of molecular epidemiology, such as identifying target antigens and understanding the parasite's genetic variability. This may go a long way towards reducing the impact of infection. Therefore, molecular epidemiology of *H. contortus* contributes to the overall understanding of the parasite's biology, evolution, and interactions with hosts. It allows researchers and veterinarians to make informed decisions about developing effective management and control strategies that lead to better animal health and productivity.

2.6. Diagnosis of haemonchosis

Correct parasite identification and disease diagnosis provide key information for strategic management, treatment, and control of GIN infections (Kandil *et al.*, 2018). The main GIN identification and associated disease determination include clinical manifestations, immunological-based, morphology, and molecular diagnostic protocols.

2.6.1. Clinical signs

Clinical diagnostic protocols are employed to establish the clinical manifestation of parasitic gastroenteritis (Patel *et al.*, 2019). These diagnostic methods are useful in disease surveillance and regular flock health management and monitoring systems. Clinical signs and animal health history are used to prescribe an appropriate action. The animal’s emaciated state, anaemia, and the accumulation of fluid in sub-mandibular tissues define small ruminant gastroenteritis (Pedreira *et al.*, 2006; Wang *et al.*, 2018). Though clinical diagnosis,

clinical signs and animal history may be sufficient to determine mitigation in a veterinary setting, a laboratory test may be sourced as a confirmatory test (Eysker and Ploeger, 2000). In surveillance and epidemiological studies, clinical and laboratory tests complement each other for monitoring herd health against helminthiasis (Roeber *et al.*, 2013).

2.6.2. Hematological-based assays

The protocols are based on the high pathogenic activity portrayed by *H. contortus* in small ruminants exhibiting marked anaemia. Two main haematological-based assays used to diagnose haemonchosis severity are the blood-packed cell volume (the hematocrit test) using red blood cell values and eye-lid colouration method called the 'FAfa MAlan CHArt' (FAMACHA). (Malan, *et al.*, 2001; Flay, *et al.*, 2022). FAMACHA primarily works by assessing the level of aneamia due to blood feeding by *Haemonchus*. The procedure is based on the relationship between anaemia intensity and the paleness of the ocular membrane. The method utilises the score chart, with values from 1 to 5, matching colours ranging from red/pink (normal-1) to clear white (highly anaemic-5) (Besier, *et al.*, 2016). FAMACHA was introduced to help small ruminant farmers easily detect haemonchosis and decisions about animals that need treatment (Tariq, 2015). The tests are essential for individual animals' health and are used for selective treatment for haemonchosis rather than treating the whole flock to manage resistance (van Wyk and Malan, 1988; Bath, 2016). These methods perpetuate the provision of parasites unexposed to drugs (refugia) also known to partake in delaying the development of resistance by maintaining parasite genetic diversity in the pastures.

2.6.3. Faecal egg counts

Morphological identification and characterization of GIN eggs, larvae, and adult stages have traditionally been the most common and routinely used diagnostic methods. Faecal egg counts have been used widely to confirm the presence and relative loads of parasitic infections using a McMaster slide. A similar principle is applied in the detection of drug efficacy and AR development by the faecal egg count reduction test (FECRT) assay. Counts of larvae recovered from pastures may also be used to determine the presence and estimate the pasture contamination levels. The McMaster technique of faecal egg count (FEC) protocol may be applied with different modifications The principle of the protocol is

identification based on the parasite's size, morphological appearance, shape, and presence of internal inclusions of worm eggs or protozoa oocysts in the faecal sample. Shorb (1939), Soulsby (1982) and Indre *et al.* (2010) provided diagrammatic identification guides for several GIPs together with their common host animals to ease diagnosis. This method is the most commonly used because it is low-cost, requires basic equipment, and is the easiest to perform, especially in disease diagnostic settings (Roeber *et al.*, 2013). Notwithstanding, the identification and differentiation of most pathogenic GINs are difficult using standard diagnostic faecal floatation techniques because of the similarity of eggs in size and morphology, which makes results arbitrary and highly inconclusive (Heise *et al.*, 1999; van Wyk and Mayhew, 2013).

2.6.4. Morphometric identification of third-stage larvae

The coproculture protocol entails the production of third-stage larvae by incubation of nematode eggs in faecal matter (van Wyk and Mayhew, 2013). The hatched larvae may then be harvested, isolated, and identified morphologically and morphometrically under the microscope to determine their genus or species identity. This method is usually used in diagnostic laboratories as a follow-up for identified nematode eggs. GIN eggs are usually challenging to determine their species due to their close resemblances (van Wyk and Mayhew, 2013). Upon determination of strongyle egg presence in the ruminant's faeces, further characterization to identify the nematodes to genus or species level is done by larval culturing and morphometrical analysis (van Wyk and Mayhew, 2013; Roeber *et al.*, 2013).

2.6.5. Pasture larval counts

Knowledge about the bioecology and off-host life cycle parameters of GIN is important for general parasite dynamics and diagnostics (Eysker and Ploeger, 2000; Bolajoko and Morgan, 2012). GIN eggs from animal faeces are passed onto the environment, where they hatch into larvae. The pasture larval counts assay involves herbage collection, larval isolation, identification, and counting (Roeber *et al.*, 2013). The grass is cut using a knife or a pair of scissors and taken to the laboratory where larvae are isolated, identified, and counted. The protocol is used to study parasites' relative intensity, off-host behaviour, spatial distribution, and seasonality (Bolajoko and Morgan, 2012). Moreover, the method investigates the interaction between bioecological and physiological parameters of parasites.

Notwithstanding its usefulness and practicality, pasture larval counts can be a laborious task to undertake (Roeber *et al.*, 2013; Eysker and Ploeger, 2000).

2.6.6. ELISA (Enzyme-Linked Immunosorbent Assay)

Enzyme-linked immunosorbent asssay (ELISA) is a broadly used method to detect the presence and quantify specific antigens or antibodies ELISA can be adapted to detect associated with an infection (Qamar and Maqbool, 2012; Kandil *et al.,* 2016). In this approach, antibodies or antigens are immobilized on a solid support and allowed to react with the sample **(**Flay *et al.*, 2022**)**. As an alternative to traditional faecal egg counts, ELISA is reliably used for the early detection of infections and can be applied to numerous tests simultaneously (Kandil *et al*., 2017). Sero-prevalence of disease can easily and timely be established using ELISA (Gowda, 2016). However, Hassan *et al*. (2019) submitted that using faecal egg counts and ELISA yielded dependable results. ELISA is commonly used in medical diagnostics, research laboratories, and various industries (Qamar and Maqbool, 2012).

2.6.7. Postmortem Examination

During hyperacute infections, animal mortalities occur and postmortem examination of the abomasum usually reveals the Barber's pole worm. Morphological features of adults are used to identify *H. contortus* at the genus or species level (van Wyk and Mayhew, 2013). Using adult morphology for differential diagnosis and characterization of closely related trichostrongylids is complex, time-consuming, and requires highly trained expertise to execute (Heise *et al.*, 1999). A simplistic identification of *H. contortus* revealed the female to be 18–30 mm long with a characteristic "barber's pole" look of twisting white ovaries and uteri around a reddish blood-filled intestine. The males are relatively shorter, measuring 10–20 mm, and present an even reddish-brown color with a tail ending with a bursa (Widiarso *et al.*, 2018; Gareh *et al.*, 2021). Nevertheless, comparative morphological identification of GIN adults and larvae provides an important approach in research and laboratory diagnostics despite their relatively low reliability and sensitivity in characterizing GINs to species and sub-species levels (Roeber *et al.*, 2013).

2.6.8. Molecular diagnosis

The ability to accurately identify all stages of a parasite's life cycle through molecular diagnostics has substantial implications for the diagnosis, treatment, and management of parasitic diseases in light of recent advances in parasite biology, epidemiology, and ecology (Gasser, 2006). There are various limitations on the sensitivity and/or specificity associated with conventional parasite diagnosis that includes being time-consuming, high-cost, and labour-intensive (Hoorfar *et al.*, 2004; Wang *et al.*, 2017). DNA-based diagnostic protocols led to the discovery of new, accurate, sensitive, relatively fast, and focused methods applied in parasitology. Some disadvantages of conventional techniques can be solved by techniques that use the enzymatic amplification of nucleic acids (Gasser, 2006). The DNA-based technique (PCR) is a technique that has been used successfully in many biological fields to selectively amplify target DNA segments from complex genomes (Allendorf *et al.*, 2010; Mangkit *et al.*, 2014). The preparation process of samples for PCR analysis depends on the sample type and the primary goal of the researcher since no one method works for all sample matrices and/or applications (Hoorfar *et al.*, 2004). Rådström *et al.* (2004) summarised sample preparation in molecular diagnostics into three main goals, which are to (i) concentrate the target DNA locus of interest for subsequent PCR, (ii) remove potential PCR inhibitors, and (iii) produce a homogeneous sample for targeted and sensitive enzymatic amplification. Different variants of PCR, such as conventional PCR, quantitative real-time PCR (qPCR), and reverse transcription PCR (RT-PCR), can be employed to detect and quantify *H. contortus* DNA in clinical samples like faeces or tissues (Samson-Himmelstjerna *et al.*, 2002; Zarlenga *et al.*, 2016). Specific primers designed to target unique regions of the parasite's DNA are used, and amplification of such regions indicates the presence of the parasite (Allendorf *et al.*, 2010). An alternative to using cultured L_3 and saving time on doing a morphological assessment is antemortem PCR-based identification of faecal eggs in which eggs are isolated without prior purification but isolating DNA from the entire faeces (Roeber *et al.*, 2013).

2.6.9. Loop-Mediated Isothermal Amplification (LAMP)

LAMP is an isothermal DNA amplification method that can detect target DNA sequences with higher specificity and sensitivity than normal PCR (Melville *et al.*, 2014). It operates under constant temperature conditions and produces visible results, often turbidity or color

change, without specialized equipment (Notomi *et al.*, 2000). LAMP assays have been developed for *H. contortus* detection, allowing for rapid and field-friendly diagnostics and the detection of SNPs responsible for benzimidazole resistance in the isotype-1 β-tubulin gene (Tuersong *et al*., 2020). LAMP is relatively faster than conventional PCR thus saving time (Parida *et al*., 2008). Consequently, the fast, sensitive and accurate LAMP protocol has also been useful in epidemiological surveys of multicellular parasites and protozoan infections (Deng *et al.*, 2019).

2.6.10. Multiplex Assays

The multiplex assays allow the simultaneous detection of multiple pathogens in a single sample reducing the need for multiple tests (Roeber *et al*., 2013). Multiplex PCR or multiplex LAMP can be designed to detect not only *H. contortus* but also other common GIPs (Zarlenga *et al.*, 2001). This is particularly useful for conducting comprehensive diagnostic assessments and could also determine the percentage of genera or species present in a mixed-species infection (Roeber *et al*., 2013; Rashid *et al*., 2018). Multiplex assays provide strong, efficient, and high-throughput testing abilities that make them dependable in complex data for diagnostics and research initiatives.

2.6.11. Next-Generation Sequencing (NGS)

Next-generation sequencing also known as high-throughput sequencing, is a revolutionary technology that has transformed the field of genomics by enabling rapid and cost-effective sequencing of DNA and RNA (Baltrušis *et al.*, 2018). NGS technologies, such as Illumina sequencing, can be used to directly sequence and analyze DNA from clinical samples (Antonopoulos *et al*., 2024). This approach can provide a comprehensive understanding of the parasite population, including potential drug-resistance mutations. It is especially valuable for research purposes and understanding the genetic diversity of *H. contortus* populations (Melville *et al.*, 2014).

2.6.12. Digital PCR (dPCR)

Digital PCR is a method that partitions a sample into thousands of individual reactions, allowing for absolute quantification of DNA molecules (Elmahalawy *et al*., 2018). The technique provides accurate quantification of low-abundance DNA targets, which can be

particularly useful in cases of light parasitic infections (Baltrušis and Höglund, 2023). The dPCR assays have been effectively used in surveillance, diagnostics, enumeration of mixed parasites, and AR determination (Tsokana *et al*., 2023).

2.6.13. Molecular Characterization

Molecular characterization of GINs has lately gained popularity in parasite diagnostics. Molecular protocols are used in tracer studies, phylogenetics, population genetics, and epidemiology (Yin *et al.*, 2016). These assays used specific genetic markers for DNA amplification and/or sequencing (Roeber *et al.*, 2013). The DNA region of interest should have adequate variations for differentiating and identifying the parasites to the appropriate taxonomic level. Molecular determination identifies within-population variants such as subspecies, genotypes, or "strains." Complete genomic DNA protocols are more precise but relatively expensive. Short fragments continue to determine allele frequencies, population structures, and conservation genetics (Allendorf *et al.*, 2010; Wit *et al.*, 2017). The use of partial genomic DNA and sequencing is developing the knowledge base in molecular applications.

Internal transcribed spacer genes of ribosomal (ITS-1 and ITS-2-rDNA) DNA have been used for trichostrongylid identification projects (Stevenson *et al.*, 1992; Mangkit *et al.*, 2014; Chaudhry *et al.*, 2015; Zhang *et al.*, 2016; Kandil *et al.*, 2018). Molecular markers have specific genomic conserved regions unique to individual species. The ribosomal DNA of eukaryotes comprises three rRNA genes (18S, 5.8S, and 28S) separated by ITS-1 and ITS-2, an intergenic spacer (comprising an external transcribed spacer and a non-transcribed spacer), and the external transcribed spacer (ETS) (Khanal, 2018) (Figure 2.5). The intra-specific genetic variation of ITS-2 sequences is low; hence, appropriate tool for precise identification between closely related species (Mangkit *et al.*, 2014). These gene segments are stable and are called the housekeeping genes. The ITS-2 gene of the ribosomal DNA (rDNA) was also targeted to study population genetics due to its high mutation rates (Heise *et al.*, 1999; Avramenko *et al.*, 2015). To demonstrate the sensitivity of molecular protocols, Amarante *et al.* (2017) designed two primer sets that could not only differentiate *H. contortus* from *H. placei* but could also differentiate between the hybrid species. Moudgil *et al.* (2022), on the other hand, successfully used the 28S–18S rRNA intergenic spacer for *H. contortus* identification. Mangkit *et al.* (2014) sequenced male *H. contortus* ITS-2 from

several populations in Thailand and managed to determine genotype diversity and nucleotide diversity. Kandil *et al.* (2018) managed to demonstrate that, apart from investigating genetic diversity and gene flow patterns in *Haemonchus* species, the taxonomic classification of different genotypes could be investigated. Powers *et al.* (1997) reiterated that the ITS genes were multipurpose genetic markers capable of diverse applications that include taxonomic identity, phylogenetic analysis, and genetic population structures.

Genomic characterization by sequencing has been endorsed as a new assay for confirmation of nematode species in diagnosis, phylogenetics, and population studies (Dey *et al.*, 2019). The availability of the whole genome (370 Mb) sequence of *H. contortus* by Laing *et al.* (2013) revolutionised genotyping and made molecular identification of this parasite a simple undertaking. Palevich *et al.* (2019a) also assembled *H. contortus* PCR-free whole genome with 465 mb encoding 22,341 genes, which also provides valuable genomic referencing for a wide range of applications.

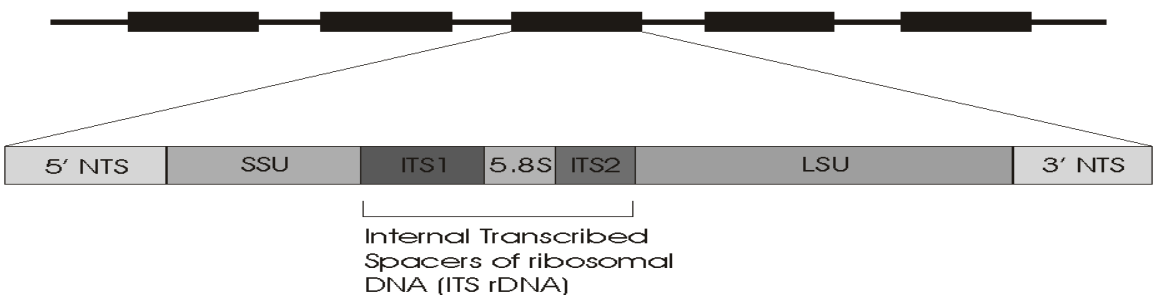

Figure 2.5: Ribosomal DNA structure showing repeat units in black boxes. The structure depicts non-transcribed spacers (NTS), a small subunit (SSU), two internal transcribed spacers genes (ITS-1 and ITS-2) with the 5.8S gene between them and the large subunit (LSU) (adapted from Troell, 2006 unpublished Figure. not to scale).

2.7. Molecular Diversity in Gastrointestinal Nematodes

Genetic variation among individuals in a population is a phenomenon core to Darwin's theory of evolution through natural selection and is responsible for subsequent variations in phenotypes (Fleming *et al.*, 2006; Ewens *et al.*, 2013). The evolutionary success of GINs has been determined by their exploitation of a wide range of lifestyles and choices of hosts

while, in the process, adapting to complex environmental conditions comprising morphological, physiological, immunological, and genetic transformations (Besier *et al.*, 2016; Salle *et al.*, 2019). Consequently, high levels of variability within and among populations are also regarded as fitness indicators for their ability to respond to daunting life-threatening challenges such as diseases, drugs, and unfavorable environmental conditions, whereas low variation levels limit a species' ability to adjust to threats for either long or short periods (Amos and Harwood, 1998). *Haemonchus contortus*, though historically originating from Africa, has since spread globally, infecting ruminants and becoming the most prevalent and economical nuisance to livestock production (Gilleard and Redman, 2016). To demonstrate the versatility of molecular analysis in the determination of diversity, the 231 bp analysis of the ITS-2 gene of *H. contortus* was found in four distinct groups of goats, sheep, and cattle, conversely, there was genetic variability even among the sympatric parasite populations in Tunisia (Akkari *et al.*, 2013).

The mitochondrial DNA (mtDNA) has also been reliably used as a molecular marker for evolutionary and molecular population studies for almost three decades (Galtier *et al.*, 2009). The mitochondrion has a unique genome different for all species of organisms, hence its success when used as a genetic marker (Gasser *et al.*, 2008). The mtDNA has characteristically high mutation rates and shows heteroplasmy, which are ideal for evaluating close relatives and assessing subspecies variations. The protocol also examines levels of mtDNA polymorphisms in assessing levels of relationships between individuals in a population and extrapolates parameters such as the standard analysis of variance (DeSalle *et al.*, 2017). This molecular marker is therefore ideal for this task because of its relatively smaller size, which makes it easy to manipulate.

Two commonly used mitochondrial DNA genetic markers for the characterization of *H. contortus* are the cytochrome oxidase subunit 1 gene (mtDNA CO1) and mitochondrial nicotinamide dehydrogenase subunit 4 (nad4) (Prichard, 2001; Basil *et al.*, 2012; Kandil *et al.*, 2016). These molecular marker analyses have been commonly utilized to examine *H. contortus* population genetics and patterns of gene flow across the globe (Australia, Brazil, China, Italy, Malaysia, Pakistan, the USA, and Yamen) (Yin *et al.*, 2016). The mitochondrial DNA cytochrome oxidase subunit-1 gene has been used to evaluate the genetic diversity of *H. placei* and *H. contortus* in a variety of animals, including cattle, sheep, goats, and

buffaloes, from two regions in Brazil (Brasil *et al.*, 2012). Kandil *et al.* (2018), while investigating gene flow using the mitochondrial CO1 gene, discovered some genetic variation in isolates from different domestic ruminants in Egypt. The availability of numerous genomes at GenBank makes molecular work done on genetic variations within the nematodes doable due to comparisons between the parasites in question and two or more reference genomes (Emery *et al.* 2016).

The mtDNA genomes have been successfully used to study genomic diversity and population dynamics globally, and several reports have been published on GINs (Gilleard and Redman, 2016). The whole genome of *H. contortus* has been sequenced, providing invaluable data for numerous applied research and post-genomic applications for strongyle parasites (Laing *et al.*, 2013). Genetic characterization and genetic sequencing have also been adopted for confirmation of nematode species in diagnosis and phylogenetic analysis (Dey *et al.*, 2019). Palevich *et al.* (2019a) successfully sequenced the complete mitochondrial genomes of *H. contortus* and *T. circumcinta*, which allowed for assessing parasite variation down to the sub-species level. Dey *et al.* (2019) demonstrated that molecular characterization of GINs and phylogenetic analysis may assist in comprehending resistance and its spread within the host population. Furthermore, mtDNA in population genetics and genotypic characterization has lately been used to accurately diagnose resistant and susceptible parasitic populations (Hu *et al.*, 2004).

Factors responsible for the genetic diversity and population structure of helminth parasites include population size, environmental and geographic conditions, life history, and gene flow (Gilleard and Redman, 2016). Population genomic approaches are further used to study the evolutionary and demographic history of parasite distributions, to determine trends in parasite transmission and invasion, to determine genetic loci of mutations responsible for anthelmintic drug resistance, and eventually to study the emergence and spatial distribution of drug resistance development in parasites (Gillead and Redman, 2006). Consequently, the broader use of genomes in molecular epidemiology has allowed researchers to explore the genetic basis of infections and parasitic variants within host populations. Different specific molecular techniques have been developed for special objectives for researchers, representing a variety of molecular diagnostic methods used for the identification and genetic diversity in nematode parasites of livestock.

2.8. Population Structure of *Haemonchus contortus*

The population genetic structure demonstrates the total genetic variations and the dispersal of species within and among a set of parasite populations (Gilleard and Redman, 2016). Genetic variation is perceived as an adaptation strategy that determines the survival of a population as it adjusts to changing environmental conditions (Emery *et al.*, 2016). The genetic variation in parasitic populations is influenced by factors such as parasites' evolutionary history, life cycles, population size, the host's spatial distribution, and gene flow (Archie and Ezenwa, 2011; Pitaksakulrat *et al.*, 2018). The sole dispersal of *H. contortus* is a host movement for socio-economic and pasture-seeking activities and is also a major determinant of genetic variability and gene flow among parasitic populations (Blouin *et al.*, 1995). Population studies have had two major thematic interests, in which the variation of phenotypes and that to which such traits are genetically expressed.

H. contortus is distributed in different climatic zones but is most prevalent in the tropics and subtropics (Molento *et al.*, 2016). Several studies were conducted across the globe for GINs' genetic diversity and population structure using ribosomal and mitochondrial genomes (Brasil *et al.*, 2012; Kandil *et al.*, 2018; Dey *et al.*, 2019). A high degree of intra-population diversity in *H. contortus* was perceived to originate from factors including high biotic potential, high rate of parasite transmissions, and large population size (Blouin *et al.*, 1995; Gilleard and Redman, 2016; Dey *et al.*, 2019). The population genetic structure of *H. contortus* using mitochondrial molecular markers, nad4, and mtDNA CO1, revealed there are more genetic variances within the population (> 90%) but usually low distribution among groups (Kandil *et al.*, 2018; Shen *et al.*, 2019; Dey *et al.*, 2019 Pitaksakulrat *et al.*, 2021). Molecular-based protocols used for genotyping for various applications such as research, diagnostics and genetic characterization of the GIN of ruminants are summarised in Table 2.1.

Table 2.1: Different genotyping methods used for the molecular diagnostics, characterization, and determination of genetic variation in GIN of livestock (Lymbery and Thompson, 2012).

Function	Purpose	Protocols
Differentiation beyond the species level	Systematics	Highly conserved coding regions e.g. SSU rDNA, mtDNA (e.g. NAD)
Distinction between species	Systematics/diagnosis/epidemiology	Moderately conserved regions e.g., coding mtDNA, ITS rDNA, and other loci (e.g., house-keeping genes such as GDH, TPI, HSP, Actin, etc.); mPCR, LAMP
Differentiation between intraspecific variants	Population genetics/breeding systems /host specificity /molecular epidemiology /conservation (e.g. susceptibility test on pathogens) /biosecurity issues	Variable regions e.g., allozymes, RAPD, AFLP, PFGE, PCR-RFLP, pyrosequencing, mPCR, LAMP, qPCR
Differentiation between isolates/clonal lineages/sub-genotypes of species	'Fingerprinting'/molecular epidemiology – tracking the transmission of sub-genotypes/ epidemiological factors/ competitive interactions and origin of infection	Fingerprinting techniques e.g. Mini/microsatellites, SSCP, qPCR
Genetic markers/linking phenotype and genotype	Identifying phenotypic traits of clinical and epidemiological significance, e.g. virulence, infectivity, drug sensitivity	Genotype linked to phenotype via (i) genetic map; (ii) RDA; (iii) sequencing and/or RT-PCR of genes linked to phenotypic traits

AFLP = amplified fragment length polymorphism; ITS = internal transcribed spacer; LAMP = Loop-mediated isothermal DNA amplification; mPCR = multiplex PCR; PCR = polymerase chain reaction; PCR-RFLP = PCR-coupled restriction fragment length polymorphism; PFGE = pulse field gel electophoresis; qPCR = real-time PCR; RAPD = random amplified polymorphic DNA; RDA = representational difference analysis; RT-PCR = reverse transcription PCR; SSCP = single-strand conformation polymorphism

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2.9. Control and Management of Gastrointestinal Nematodes

2.9.1. Biological Control of Gastrointestinal Nematodes

Biological control uses natural enemies or organisms to regulate the population of pre-parasitic worms to reduce their negative impact on livestock. Certain plants and fungi have shown potential in reducing *H. contortus* larvae on pastures (Larsen *et al.*, 1997). Research is ongoing in this area to develop natural alternatives to chemical dewormers. Several biological control strategies can be employed to manage GIN infections including fungi such as the genus *Metarhizium* that can be used as biopesticides against nematode larvae (Braga and de Araújo. 2014). These fungi infect and kill the larvae, helping to control their populations. The administering of these fungi to the environment can lead to their dissemination and establishment, resulting in a long-term reduction in nematode numbers (Larsen *et al.*, 1997). Biological competitors such as animals or organisms that compete with nematodes for resources can indirectly reduce nematode populations. For example, chickens or ducks can be allowed to graze in pastures, where they consume larvae, interrupting the nematode life cycle (Szewc, *et al* 2021).

2.9.2. Pasture Management

Apart from being the main source of animal feed, pasture management can also be used to control GIN through rotational Grazing. The aim of pasture management in parasite control is to provide safe grazing for animals (Fleming *et al.*, 2006). The periodical movement of animals to different pastures in the act helps break the parasite's life cycle by reducing the exposure of animals to infective larvae on the pasture for a pronounced period (Ihler, 2010). Consequently, rotating pastures is a deliberate management regime that aims to lower the chance of reinfection and allows parasite-contaminated areas by removing the host animal temporarily (Waller, (2006). Equally important rest periods may allow pastures to be without animals for a certain period reducing the number of infective larvae on the pasture (Bresciani *et al.*, 2017). Common grazing of mixed livestock species with varying susceptibilities to nematodes, and their grazing habits can impact nematode distribution and survival.

2.9.3. Selective Breeding

Genetic selection of animals that show natural resistance or resilience to helminth infections through breeding has been one way of improving resistance traits to reduce parasite burdens in ruminants (Zvinorova *et al*., 2016). Selective breeding for gastrointestinal control involves the intentional breeding of animals with specific traits related to their gastrointestinal system. This practice is commonly done in livestock and agricultural animals to enhance their overall health, digestion, and efficiency of nutrient utilization (Sweeny *et al.*, 2016). The goal is to develop animals that are more resistant to GI disorders, have better feed conversion rates, and ultimately improve the quality and quantity of food production. There are sheep breeds that are naturally more resistant to *H. contortus* infection, which helps reduce farmers' expenditure on deworming (Emery *et al.*, 2016). Breeding for resistance may eventually reduce the overall burden of the parasite or enhance animals' tolerance towards the parasite infection (Salaris *et al*., 2022). Nieuwoudt *et al*. (2002) demonstrated breeding for resistance to GIN as viable and its incorporation into mainstream integrated control programmes.

2.9.4. Nutrition and Management

Proper nutrition can enhance the animals' immune response and reduce their susceptibility to parasitic infections while undernutrition increases the risk and vulnerability of the host animal (Hall *et al*., 2012). Metabolised protein supplementation has been shown to significantly affect the level of gastrointestinal nematode parasitism in which faecal egg counts and worm burdens were reduced (Kyriazakis and Houdijk, 2006). Good management practices, such as providing clean water, shelter, and maintaining stress-free conditions, can help animals better withstand parasitic challenges and contribute to overall animal health (Sweeny *et al.*, 2016). Lesotho livestock farming is predominantly of extensive type with communal grazing though there are initiatives to encourage intensification, especially for Merino sheep production (Mahlehla *et al*., 2021; WAMPP, 2021).

2.9.5. Anthelmintic Drug Control and Vaccines

Chemotherapeutics have been the historical mainstay of preventive and curative treatment for GIN infections across the globe (Maurizio *et al.*, 2023). These drugs continue to be used and achieve substantial results though caution has been proclaimed due to imminent GIN

developing resistance. The three major classes of anthelmintic drugs used for controlling GIN in ruminants are benzimidazoles, macrocyclic lactones, imidazothiazoles (levamisole) along with tetrahydropyrimidines each class possessing a different mode of action against worms (Kwa *et al.*, 1994). Benzimidazole Inhibits microtubule formation in parasitic cells, preventing glucose uptake, leading to starvation and death of the worm, macrocyclic lactones (MLs) ivermectin, doramectin, eprinomectin, and moxidectin disrupt neurotransmission in the parasite, causing paralysis and death (Martin, 1997: Abongwa *et al.*, 2017). Imidazothiazoles such as Levamisole act as a cholinergic agonist that paralyzes the worm by continual muscle contraction (Martin, 1997). Though there are abundant drug choices in the market knowledge about the drug mode of action and appropriate dosing regimes is still a challenge among farmers (Mphahlele *et al*., 2021). Administering an appropriate dose of anthelmintic drugs based on the weight of individual animals prevents either over- or under-dosing, which may promote the development of resistance by allowing some parasites to survive and reproduce (Tariq, 2015). Consequently, some strategic combination or multi-active ingredient anthelmintic products are used to target different mechanisms of action of parasites, reducing the likelihood of resistance development (Kotze and Prichard, 2016). Targeted treatment of animals using anthelmintic drugs involves being judicious and treating only when necessary to avoid routine, frequent, or unnecessary whole-herd treatments (Maurizio *et al.*, 2023). This also entails selectively treating animals only showing clinical signs or those that manifest high parasite burdens after conducting diagnostic tests such as clinical examination or faecal egg counts. The method is both economic and reduces AR development. In some rural communities with low-resource farming communities, alternative and herbal treatments have been used as supplements and remedies instead of conventional anthelmintic drugs (McGaw and Elloff, 2008).

The extensive and uncontrolled use of anthelmintics led to the development of drug resistance creating a risk to human and animal health (Gilleard *et al.*, 2006). This drawback has also led to extensive research through the discovery of vaccines. The initial work on hidden antigens against *H. contortus* yielded earlier promising results (Newton and Munn, 1990). Such vaccines function based on continuously providing antigens that target *H. contortus* and disrupt the normal processing of nutrients by the worm, eventually tempering with growth and killing the parasite (Flemming *et al.*, 2006). Several types of vaccines including, recombinant subunit vaccines and DNA vaccines are becoming alternatives to

anthelmintics (Wang *et al*., 2017). The vaccines are good prospects because they provide sustainable protection against GIN such as *H. contortus, Ostertagia ostertagi,* and *Teladosargia circumcincta* (Lui *et al*., 2023). A vaccine commonly known as barber vax, which uses natural antigens has been manufactured against *H. contortus (*Liu *et al., 2023)*.

2.9.6. Ethno-veterinary Medicines and Indigenous Therapy

Ethno-veterinary medicines and indigenous therapy comprise alternative traditional remedies and practices mainly used by resource-poor communities as supplements to conventional anthelmintic drugs to manage various animal health problems (Luo *et al.*, 2022). Management of parasites to maintain the good health of livestock in rural communities has been mainly through the use of indigenous knowledge, which was passed from one generation to the next (Bamikole and Ikhatua, 2009; Ndlela *et al.*, 2022). The entire plant and/or parts of plants are used in solitary or in concoctions. Different farming communities developed their unique methods of control and treatment (Bamikole and Ikhatua, 2009). More than 50% of farmers who raise goats in areas of Kwazulu-Natal were recorded to exclusively use ethno-veterinary remedies to manage GIN (Ndlela *et al.*, 2022).

2.9.7. Integrated Parasite Management (IPM):

Integrated Parasite Management (IPM) for gastrointestinal parasites is an all-encompassing method of controlling parasites in livestock, pets, and wildlife while lessening the use of anthelmintics to maximise efficiency and avert AR (Maqbool et al., 2017). It is worth noting that the specific strategies and treatments may vary depending on the type of animal being treated, the severity of the infection, local conditions, and the latest scientific research. van Wyk *et al.* (1999) reiterated farmers' ignorance of AR and their hesitancy thereof, together with the usage of less effective drugs exacerbated the development of AR in farms in South Africa. Integrated Parasite Management combines various strategies, such as pasture management, anthelmintic use, and nutrition, to create a holistic approach to nematode control and prevention (Maqbool *et al.*, 2017). Regular consultation with a veterinarian or parasitologist is crucial for developing an effective and sustainable plan for controlling and treating GIN in animals. Monitoring faecal egg counts is critical in assessing parasite burdens, enabling targeted treatments based on specific infection levels, rather than relying

on routine deworming. This allows for targeted treatment based on actual needs rather than routine deworming.

2.9.8. Gastrointestinal Nematode Control in Lesotho

The major anthelmintic drugs used in Lesotho include benzimidazoles (such as valbantel and valbazen), levamisole, niclosamide, and ivermectin (Moiloa *et al*., 2020). Mahlehla *et al*., (2021) reported more than 90% of sheep farmers from Maseru and Quthing districts, in Lesotho used anthelmintic drugs and ethno-veterinary remedies. Livestock farming communities in Lesotho are mainly located in resource-poor areas including rural and mountain settlements. Moiloa *et al.* (2020) and Mahlehla *et al.* (2021) reported that most small ruminant farmers either were unschooled or attained at least primary education.

2.10. Anthelmintic Resistance Development in Gastrointestinal Nematodes

2.10.1. The Origin and Development of Anthelmintic Resistance in Small Ruminants

It was hypothesized that the alleles associated with the development of AR were historically always present at low frequencies and at random among parasite populations before the onset of anthelmintic treatment (Gilleard and Beech, 2007). Frequent drug treatment gives the resistant nematode an unprecedented reproductive advantage over the susceptible strain (Melville *et al.,* 2016). Other possibilities for the origin of AR alleles include the instant appearance of a novel resistance mutation just before the onset of treatment and gene flow from adjacent populations as a result of animal movement (Gilleard and Beech, 2007). Repeated treatment with an anthelmintic drug against GINs was also observed to result in the subsequent rise in more drug-tolerant strains (Kwa *et al.*, 1993; Beech *et al.*, 2011; Kotze and Prichard, 2016). Notwithstanding, farming communities harbour two parasite sub-populations. The first are those directly exposed to administered drugs, while the second entails the non-anthelmintic-exposed free-living stages in the environment, known as the in-refugia parasite population. Melville *et al.* (2016) observed a marked decline in parasite diversity of in-refugia and an increased AR after the whole flock was frequently treated with a benzimidazole drug. The relative numbers of these two sub-populations of parasites may have varying effects on the overall progression of AR during a particular treatment regime.

Comparatively, large in-refugia parasite populations and low drug-exposed parasites lead to slower development of resistance (Melville *et al.*, 2016; Hodgkinson *et al.*, 2019). However, other factors were reported to contribute to the rate of AR development; these include (i) time of re-infection post-treatment, (ii) administration of an insufficient quantity of drug, (iii) inappropriate drug storage, and (iv) usage of an incorrect drug as a result of a misdiagnosis (Vande Velde *et al.*, 2018; Sangster *et al.*, 2018).

The benzimidazoles, levamisoles, and ivermectin are three major drug classes used worldwide for the control of economically important livestock GINs (Kwa *et al.*, 1994; Tsotetsi *et al.*, 2013). The development of AR is a common phenomenon among GINs, especially in extensive anthelmintic treatment areas (Zhang *et al.*, 2016; Ali *et al.*, 2019). AR is a genetically transmissible trait in which normal susceptible populations of parasites eventually develop tolerance and survive a normally effective drug dose (Fleming *et al.*, 2006). Consequently, AR development for such drug groups rendered parasite management and control difficult, resulting in escalated economic losses in the livestock industry (Gilleard and Beech, 2007; Smith, 2014; Selemon, 2018). Inevitably, the benzimidazole group is the most widely available, commonly used, and relatively cheaper of all drug groups (Zhang *et al.*, 2016). Several molecular changes associated with AR development were discovered in beta-tubulin isotype-1 (benzimidazole), glutamate and GABA-gated chloride channels (macrocyclic lactones), and nicotinic acetylcholine-gated cation channels (Levamisole) (Beech *et al.*, 2011). Molecular markers were eventually developed to detect these changes in GINs for AR development.

The benzimidazoles are designed to disrupt the core functions of microtubules within the cell. Microtubules are filamentous organelles made from linearly arranged proto-filamentous alpha and beta-tubulin heterodimer subunits (Sangster *et al.*, 2018; Santana-Molina *et al*, 2022). These structures form the cytoskeleton, provide support, and maintain the cellular structure of eukaryotic cells (Lacey, 1988). The alpha- and beta-tubulin proteins are components of tubulin globular proteins and the largest protein groups in eukaryotes that are core to cellular functions (Lacey, 1988). Microtubules are also relevant to the origin and diversification of life (Santana-Molina *et al.*, 2022). The benzimidazole drugs bind with the beta-tubulin monomers in a parasite to form a tubulin-benzimidazole complex that then inhibits the development and replenishment of microtubules (Sangster *et al.*, 2018; Lacey,

1988). As benzimidazoles bind to the helminth tubulin target, they interfere with the function of microtubules by weakening them in providing for intracellular transport channels, nutrient absorption, and cytokinesis (Lacey, 1988; Straight and Field, 2000; Fissiha and Kinde, 2021). The parasites therefore starve, become incapacitated, and may eventually die. A strong association was established between the reduction of microtubules in the intestinal cells of susceptible parasites after extensive benzimidazole drug treatment and the opposite happening in resistant strains (Sangster *et al.,* 1985). Furthermore, work on *Caenorhabditis elegans* revealed that variations observed in the beta-tubulin gene were responsible for AR in the benzimidazole drug group (Saunders *et al.,* 2013; Roberts *et al.,* 2013). A decline in benzimidazole binding to beta-tubulin in *H. contortus* was confirmed to be associated with AR development (Lubega and Prichard, 1991).

The three codons of benzimidazole resistance-associated single nucleotide polymorphism (SNPs) are located in exons 4 and 5 of the beta-tubulin isotype-1 gene. Figure 2.7 is a graphical presentation of a complete beta-tubulin isotype-1 gene, which comprises nine exons and 10 introns (Kwa *et al.,* 1993). Exons are the coding regions of the genes responsible for the information necessary to produce beta-tubulin protein, while introns play various roles in gene regulation. The initial molecular investigation on mutations in the beta-tubulin gene portrayed a consistent change on codon 200, depicting a substitution of phenylalanine (TTC) to tyrosine (TAC) (Silvestre and Cabaret, 2002). Later on, other SNPs were also discovered in codons 167 (TTC to TAC) and 198 (GAA to GCA) (Silvestre and Cabaret, 2002; Kwa *et al.,* 1994). The beta-tubulin gene was also found to possess conserved sites and may also be regarded as a housekeeping gene capable of being used in the identification and characterization of GIN species and other associated variants (Santana-Molina *et al.,* 2022).

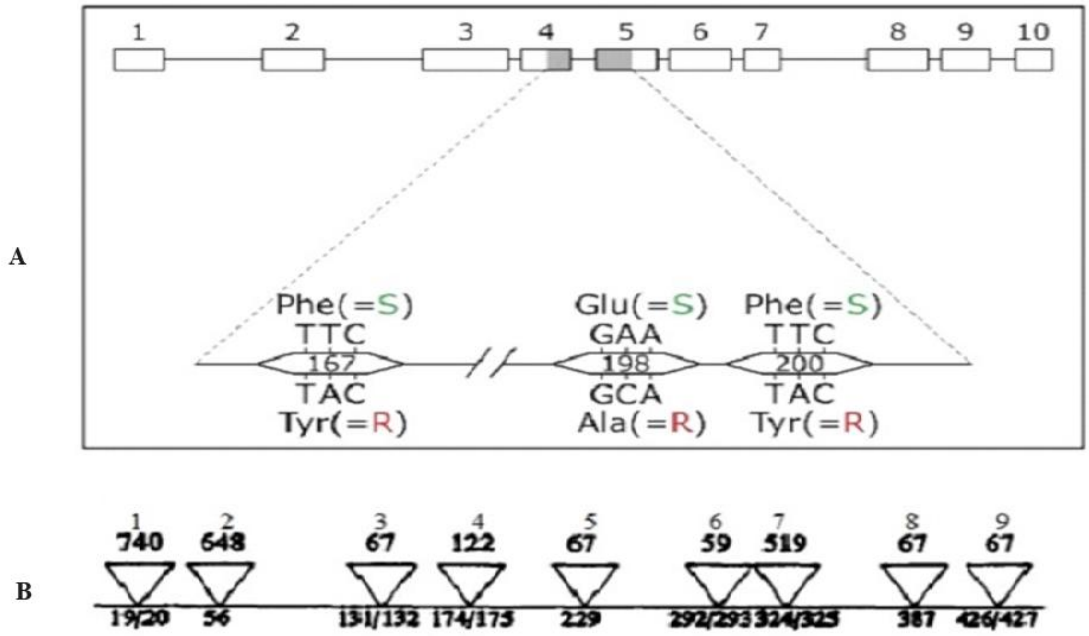

Figure 2.6: The structural presentation of the beta-tubulin isotype-1 gene depicting the structure of the benzimidazole-associated drug binding region with exons and introns is also shown in the diagram. **A.** Three defined point mutations responsible for AR are found on exon 4 (codon, 167) and 5 (codons, 198 and 200). **B.** The base pair number of the introns is written on the triangles. Numbers below the triangles show the location of introns in the gene. Modified from Kwa *et al*. (1993) and Baltrušis (2022).

Several nematodes have been reported to develop drug resistance to benzimidazoles, including *Ascaris lumbricoides in humans, Teladosargia circumcincta, Trychostrongylus columbriformis,* and *Cooperia* spp. in ruminants (Jones *et al.*, 2022; Von Samson-Himmelstjerna *et al.*, 2007). High levels of genetic diversity among Trichostrongylids are attributable to immense population sizes, consequently exhibiting high mutation rates (Dey *et al.*, 2019; Blouin *et al.*, 1995). To demonstrate their capacity to survive, *H. contortus* developed resistance to ivermectin only three years after introduction, and other parasites exhibited multiple resistances to drugs such as fenbendazole and closantel, except levamisole (van Wyk and Malan, 1988). Table 2.2 presents a summary of different anthelmintic drug groups, depicting their modes of action and the estimated time of resistance appearance in animals. Macrocyclic lactone drug group members such as ivermectin function by opening the chloride channels and causing starvation in GIN (Fissiha

and Kinde, 2021). Correct diagnosis of infective parasites and the ability to detect AR are important steps towards strategic parasite management and control (Calvete *et al.*, 2014).

2.10.2. Detection of Anthelmintic Resistance in Small Gastrointestinal Nematodes of Ruminants

Three methods used for the detection of efficacy and anthelmintic resistance used are \namely, the faecal egg count reduction test (FECRT), egg hatch assay (EHA), and larval development test (LDT), with varying pros and cons on their reliability, consistency, result interpretation, and cost (Chaudhry *et al.*, 2015). One of the most reliable *in vitro* methods of AR detection to date is the egg hatch assay, sanctioned by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles *et al.*, 2006; Calvete *et al.*, 2014). The protocol relies on the ovicidal properties of benzimidazole and on the capability of eggs of resistant strains to hatch at higher drug concentrations than those from susceptible strains (Mphahlele *et al.*, 2021). Thiabendazole is a common drug used for diagnostic protocols because of its solubility and ability to be diluted through serial dilution. The protocol eventually estimates concentrations of the drug that inhibit the development and hatching of more than 50% of the parasite eggs (ED). Lindberg and Vatta (2006) reported the emergence of AR due to the extensive use of anthelmintic drugs in resource-poor farming systems in South Africa. The emergence of AR and its daunting economic implications require reliable resistance detection methods and their quest to minimize the escalating losses to the industry. To address the inherent shortcomings of conventional methods in AR detection such as the time-consuming, high drug cost, low sensitivity, and labor-intensive, researchers have continually developed accurate and more sensitive protocols, hence PCR molecular markers (von Samson-Himmelstjerna, 2006).

Gastrointestinal nematodes possess paired chromosomes at each gene locus providing a basis for AR detection protocols and showing allelic variations (Fissiha and Kinde, 2021). These alleles are responsible for AR known to exist within the nematode population before being expressed phenotypically (Emery *et al.*, 2016). With resistance-conferring alleles recessive (RR), resistance in a population increases with homozygous recessive genotypes, with a heterozygous genotype (SR) susceptible to a drug of interest (Seesao *et al.*, 2017). The development of resistant alleles increases with the introduction and continued application of anthelmintic drugs within a parasite population (Silvestre and Humbert, 2000). The

expression of this phenotype emanates from genomic changes in the allelic structure of specific genes.

Initially, allelic changes at codon 200 of the beta-tubulin isotype-1 gene resulted in SNP in which phenylalanine (TTC) in susceptible worms was replaced by tyrosine (TAC) in resistant worms. These protocols are used to determine the genetic loci of mutations encoding for resistance to benzimidazoles (Seesao *et al.*, 2017). The detection of benzimidazole resistance-associated SNPs by molecular means in *H. contortus* was performed through the amplification and analysis of the beta-tubulin isotope-1 gene (Chaudhry *et al.* 2015). Identification of mutations in the beta-tubulin isotope-1 gene of *H. contortus* in codons 200 (TTC to TAC), 167 (TTC to TAC), and 198 (GAA to GCA) depict the detection of AR (Chaudhry *et al.*, 2016; Kumar and Singh, 2017). AR detection by an allele-specific protocol works by genotyping resistant (RR) or susceptible (SR and SS) nematodes. The allele-specific PCR protocol for the determination of codon 200 of the beta-tubulin isotype-1 gene, which is capable of estimating the proportion of genotypes on codon 200 in trichostrongyloid populations, was developed by Humbert *et al.* (2001). Nested PCR locates codons 200 (TTC to TAC), 167 (TTC to TAC), and 198 (GAA to GCA), which designate the development of AR in *H. contortus* (Kumar and Singh, 2017). The Allele-specific PCR (AS-PCR) protocol uses allele-specific primers to detect SNPs along the length of a gene sequence (Coles *et al.*, 2006). The method may also be used to determine the frequency of alleles within parasite populations.

Table 2.2: Anthelmintic drugs of ruminants and summary of their mode development of resistance to the drugs (De Graef *et al*., 2013).

Anthelmintic drug class	Mode of action	Generic drug name	Introduced to the market	First report of resistance	Reference
Heterocyclic compounds	Obstructive dopaminergic transmission	phenothiazine	1940	1957	(Leland *et al.*,1957)
	Agonist of the inhibitory GABA-receptor	Piperazine	1954	1966	(Drudge *et al.*, 1988)
Benzimidazoles	Bind to beta-tubulin and inhibit the development of microtubules. Prevents glucose uptake, protein secretion, and microtubule production, with subsequent starvation of the parasite	Thiabendazole	1961	1964	(Drudge *et al.*, 1964)
		Cambadazole	1970	1975	(Berger, 1975)
		Oxibendazole	1970	1985	(Drudge *et al.*, 1985)
		Mebendazole	1972	1975	(Berger, 1975)
		Albendazole	1972	1983	(Cawthorne and Whitehead, 1983)
		Fenbendazole	1975	1982	(Boersema and Lewing- van der Wiel, 1982)
		Oxfendazole	1976	1981	(Le Jambre *et al.*, 1981)
		Triclabendazole	1983	1998	(Mitchell *et al.*,1998)
Imidazothiazoles and Tetrahydopyrimidines	Causes agonistic action of acetylcholine causing spastic paralysis of the nematodes. Paralyzed nematodes are rapid removal of present worms.	Levamisole	1970	1979	(Sangster *et al.*,1979)
		Pyrantel	1974	1996	(Chapman *et al.*, 1996)
		Oxantel	1976	-	-
		Morantel	1970	1979	(Sangster *et al.*,1979)
Macrocyclic lactones	Causes an opening of glutamate-gated chloride channels (GluCI). resulting in elevated CI-ion influx into nerve cells, causing flaccid paralysis in nematodes	Abamectin	Late 1970's	2001	(Wooster *et al.*, 2007)
		Ivermectin	1981	1988	(van Wyk and Malan, 1988)
		Moxidectin	1991	1995	(Leathwick, 1995)
		Doramectin	1993	2007	(Borgsteede *et al.*,2007)

		Eprinomectin	1996	2003	Loveridge *et al.*,2003)
Amino-acetonitrile derivative	Mode of action comprises a nematode-specific clade of acetylcholine receptor subunits.	Monepantel	2009	-	-
	Agonist of cation channels	Derquantel	2010	-	-

Another method of AR detection used in molecular work is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), in which a PCR amplicon is digested with a specific restriction enzyme capable of producing distinct polymorphic fragments of interest (Fissiha and Kinde, 2021; Tiwari, 2006). In this protocol, restriction enzymes target several specific bases on the gene at which they fragment the DNA (Beech *et al.*, 2011). Doyle *et al.* (2022) also provided a detailed understanding of the genetics of AR development, in which they demonstrated how its distribution among parasite populations could be traced, alternatively enhancing the effectiveness and sustainability of anthelmintic management and control.

2.10.3. Management and Control of Anthelmintic Resistance Development

The effective control of GIN was achieved globally through integrated approaches that strategically combined factors such as anthelmintic drug administration, range management, and bioactive forages (Rahmann and Seip, 2007). Molento *et al.* (2016), in Brazil, also reported growth in animal products after the introduction of broad-spectrum drugs. The use of combination drugs with different active ingredients on nematodes and the introduction of "refugia" by keeping a portion of the parasite's population unexposed to anthelmintic drugs were recommended (Vande Velde *et al.*, 2018; Hodgkinson *et al.*, 2019). These strategies are meant to delay the development of resistance and ultimately optimize treatment for efficient and effective helminth control. Jabbar *et al.* (2006) contested that the escalation of GIN resistance to anthelmintic drugs was a result of failed helminthic control programs. Multiple ARs in GIN originated and spread as a result of the persistent use of anthelmintics by farmers without basic knowledge of dosage regimes and proper administration. van Wyk *et al.* (1999) reiterated farmers' ignorance of AR and their hesitancy thereof, together with the usage of less effective drugs, which exacerbated the development of AR in farms in South Africa. Moiloa *et al.* (2020) reported unsatisfactory GI parasite control among goat farmers in Lesotho due to marked disparities in their ignorance of parasites, their socioeconomic status, low access to essential services, and indiscriminate use of drugs. The exhibition of overdispersion of parasites within the host population can be investigated to conceptualize their distribution, intensity and prevalence. Overdispersion is a phenomenon in ecology and epidemiology that describes an uneven distribution pattern where some hosts in a population have a higher number of parasites than expected, while others have very few.

Researchers may take advantage of this concept by investigating the production of convenience in which inexpensive and accurate on-farm selective treatment is prioritised (Pfukenyi and Mukaratirwa, 2013).

CHAPTER 3

Barriers and drivers associated with gastrointestinal nematode control in smallholder Merino sheep farms in Lesotho

3.1. Introduction

Small ruminant farming in Lesotho exhibits a low-input farming system with characteristic transhumance pastoralism (Obioha, 2018). The majority of farms are small-scale to medium-sized and predominantly keep mixed farm animals grazed on communal rangelands (Bukhari and Sanyal, 2011). Cattle, goats, horses, donkeys, and pigs are reared mixed with Merino sheep in various species proportions and combinations in Lesotho. Small ruminants are the most commonly found in resource-poor communities including the landless nomadic and rural populations worldwide (Kumar and Roy, 2013). Comparably, Merino sheep is the dominant animal breed in Lesotho, bred for wool exports, that has contributed significantly to Lesotho's GDP for several decades and is still expected to grow (Hunter, 1987; Mokhethi *et al.*, 2015; WAMPP, 2021). Other than wool production and sales, sheep farmers also trade in meat, skin, and manure (Bekuma and Dufera, 2019). Moreover, sheep are also important in the sociocultural activities of many global communities (Obioha, 2018). Like others in low-income communities, Lesotho farmers heavily depend on communal grazing as the major source of providing animals' daily nutritional requirements (Obioha, 2018). For the sustenance of such naturally occurring resources, farmers engage in pasture management systems and food supplementation (Shivakumara and Kiran, 2019; Mahlehla *et al.*, 2021). Regional and village range management regimes divide pasturelands into sections and engage in seasonal rotations to lessen the pressures of overstocking and overuse, the "tragedy of the commons." Notwithstanding the potential economic advantages of rearing small ruminants, it is apparent that animal diseases, meagre management practices, and inadequate nutritional provisions continue to threaten the industry (Munguía-Xóchihua, 2018). Subsistence, poor-resourced farming communities, and communal grazing are extensive and inevitable systems that expose animals to an invariable risk of infection (Molento *et al.*, 2016; Kandil *et al.*, 2018; *Bekuma* and Dufera (2019). Lesotho is topographically subdivided into four distinct ecological zones, namely, the Highlands, Foothills, Lowlands, and the

Senqu River Valley. Consequently, Lesotho's climate is characterized by four marked seasons (summer, autumn, winter, and spring), in which it is extremely cold during the winters and summers are warmer with relatively more rain (Phalatsi *et al.*, 2022). Variations in topography, climatic conditions, farmers's economic status, farmer education attainment, and accessibility to key veterinary services may lead to diverse animal management and parasite control challenges among farming communities (Munguía-Xóchihua, 2018; Mphahlele *et al.*, 2021). The infection by GINs leads to clinical conditions such as emaciation, diarrhoea, poor fleece, anaemia, weakness, dehydration, bottle jaw, and eventual death (Papadopoulos *et al.*, 2003; Besier *et al.*, 2016; Patel *et al.*, 2019).

More than 80% of farmers used anthelmintic drugs in Maseru and Quthing districts, Lesotho, with the benzimidazole group being the most predominant group (Moiloa *et al.*, 2019; Mahlehla *et al.* 2021). Control of GINs may therefore remain less effective and unsustainable because of haphazard administration and inadequate coordination (Molento *et al.*, 2016). Erroneous adherence to improper drug dosages and frequent haphazard drug administration exerts genetic selection pressure on parasites for the development of AR, manifested through decreased drug efficacy and occurrence of specific anthelmintic resistance-associated SNPs (Mohanraj *et al.*, 2017; Mphahlele *et al.*, 2021). The control strategies used by goat farmers in Lesotho to manage GINs have been ineffective due to factors such as lack of parasite knowledge, low socioeconomic status, and limited access to drugs and veterinary services, all of which continue to impede livestock production in the area. (Moiloa *et al.*, 2020).

Earlier studies highlighted marked variations among small ruminant-rearing practices and parasite control strategies leading to diverse approach to animal husbandry in Lesotho (Moiloa *et al.*, 2020; Mahlehla *et al.*, 2021). The recent Wool and Mohair Promotion Project (WAMPP) initiative, among others, aims to improve management and the small ruminants' gene pool for increased wool production. It is important therefore, to investigate aspects of animal health and anthropogenic factors in small ruminant rearing and provide information that can inform stakeholders to leverage such efforts. The present study was designed to investigate the impact of anthropogenic activities on sheep farming in different ecological zones of Lesotho.

3.2. Materials and Methods

3.2.1. Study area

The study was conducted in Lesotho, a subtropical country in southern Africa that is completely landlocked by South Africa. Lesotho was formed as far back as the 1700s and 1800s due to the historical pressures of tribal wars, colonialism, and Boer expansion, which pushed the Basotho people into the steep and rugged Maluti and Drakensberg Mountain ranges (Turner, 2013; Nhemachena *et al.*, 2016). Lesotho has an overall territory of approximately 30,300 km^2. About two-thirds of the territory comprises the mountainous terrain, which is mainly composed of three grassland biomes: montane, subalpine, and alpine, which have palatable grasses that provide the majority of livestock species with their daily food requirements (Pomela *et al.*, 2000; Nhemachena *et al.*, 2016). Figure 3.1 represents a map of Lesotho depicting the study area that included areas from six districts of Lesotho. The study area is topographically stratified into four main ecological zones, namely: the Highlands, Foothills, Northern and Southern Lowlands, and the Senqu River Valley (SRV) Obioha, 2010; (Mbata, 2001). Rangelands provide communal pastures, which are a free-for-all resource. The Highlands (>2200m in altitude) areas selected comprised the Mants'onyane areas in the Thaba Tseka (-29.531186, 28.266342) district, which represented the Highlands zone. The dominant grasses in the Highlands are *Themeda triandra* and *Eragrostic curvula*, which are also palatable and nutritious (Pomela *et al.*, 2000). The lowlands (1400–1800m altitude) are mainly a grassland biome with savanna woodlands and willow brushes along the riversides (Rutherford and Westfall, 1994). The Lowlands were represented by the Southern Lowlands, which covered parts in and around Mafeteng (-29.581655, 27.316686) and Maseru (-29.489033, 27.370197) districts. The Foothills are on a patch of land with "highly visible tops," which range from 1800 to 2200 metres in altitude. They have a mixture of sporadic covers of grassland pastures and vast stretches of montane scrub woodland (Pomela *et al.*, 2000; Nhemachena *et al.*, 2016). The selected areas in the Foothills were located around the Pitseng region (-29.009722, 28.2575) in the Leribe district. SRV is represented by areas along the Senqu River that topographically stretch from 1500 to 2250 metres in altitude, also having mixed vegetation groups. The selected areas in SRV included sample sites around Quthing (-30.195345, 28.167627) and Qacha's Nek (-30.120372, 28.364839) districts.

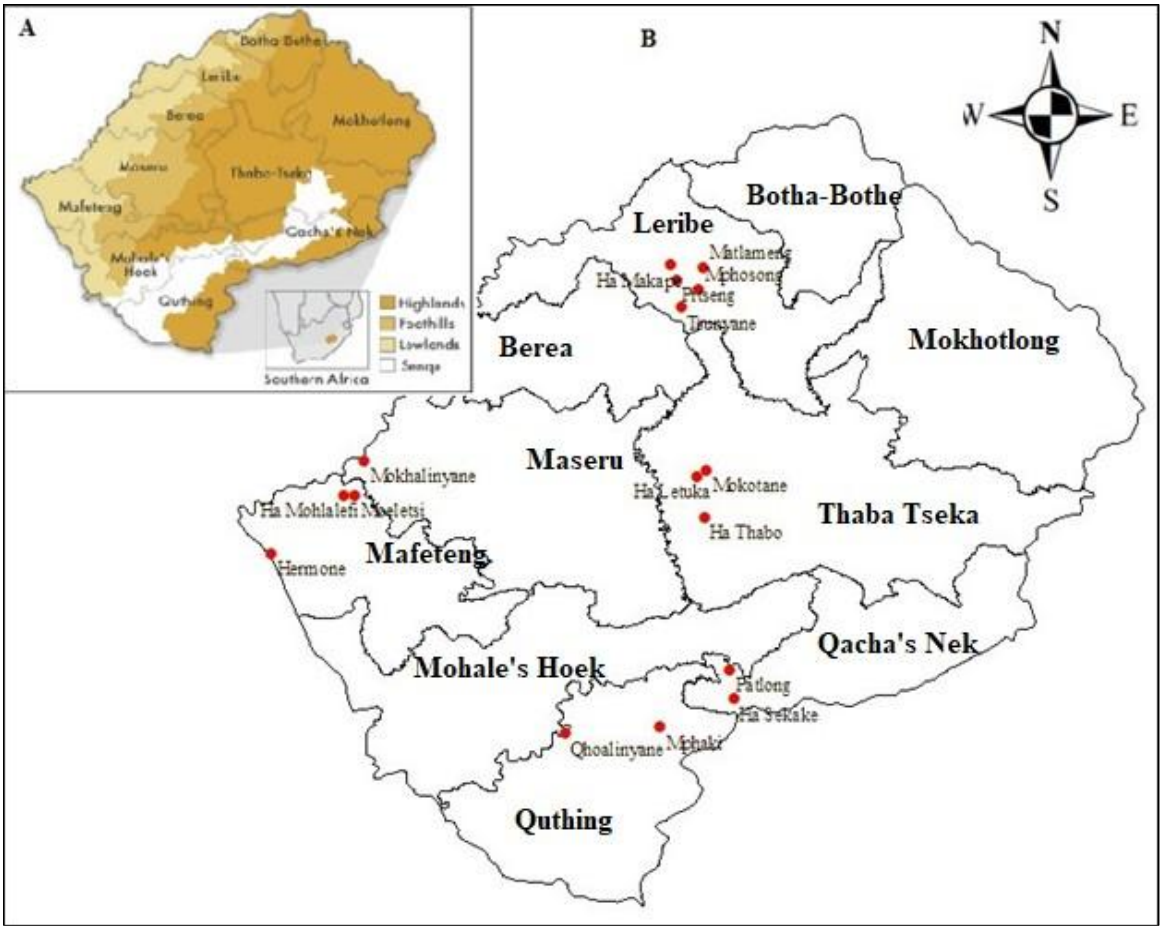

Figure 3.1: Lesotho Maps showing **A**. Topographical map of Lesotho showing four ecological zones of Lesotho. **B**. Lesotho map depicting study area with interview and collection site with and in red dots., Highlands, Foothills, Lowlands, and Senqu River Valley.

3.2.2. Experimental design

A cross-sectional questionnaire survey on the farmers' knowledge and perceptions of farm management practices, GIP infections, anthelmintic management, and control of Merino sheep was carried out in four ecological zones in Lesotho between September 2022 and April 2023. A stratified random sampling method was used, encompassing four ecological zones in Lesotho: The Highlands, Foothills, Lowlands, and SRV. Regional and local Lesotho wool and mohair growers' associations' woolshed administrators provided the farmer lists from which the sampling frame was formed and informants were selected. Sampling sites and Merino sheep farmers' lists were randomly selected amongst eight Wool and Mohair

farmers' association areas. A semi-structured questionnaire with closed- and open-ended questions was developed to gather information on farmer demographics, anthelmintic general animal management, GIN control, and drug usage among the Basotho farmers in the research area. The respondents included farm owners, farm managers, and animal herders. A total of 212 (Highlands, n = 47, Foothills, n = 51, Lowlands, n = 79, SRV, n = 35) respondents from four ecological zones were interviewed in the present survey.

3.2.3. Data analysis

The Statistical Package for Social Sciences (SPSS) package version 20.0 was used in the organization and analysis of the data collected in the study. Descriptive statistics, frequencies, and tabulations were used to collate and analyse the data. Boxplots were used to graphically display the distribution of numerical data sets in the study. The chi-square test (χ^2) was used in the present study to evaluate the associations between data sets such as farmer demographics, and farmers' knowledge, practices, and ecological zones. One-way analysis of variance (ANOVA), was used to compute if there is a difference between group means of numerical variables such as animal numbers and animal mortality between different ecological zones. A post-hoc test was subsequently employed on numeric variables to infer the separation of means, with the level of significance set at 5%. A two-tailed Pearson correlation analysis was also used to establish a linear correlation between two variables.

3.3. Results

3.3.1. Demographic status of Merino sheep farmers

The social demographics of Merino sheep farmers (n = 212) in Lesotho are presented in Table 3.1. The results portray (n = 165, 77.8%) male farmers as the dominant gender in sheep rearing. The Chi-square test revealed no significant association between the ecological zones and gender ($\chi^2 = 4.753$ and $p = 0.243$). However, the Lowlands were the most male-dominated area registering 26.4% of the overall male respondents in the present study. Age was categorized into six groups of which the 66+ age constituted 25% of all respondents while there was only one farmer in the 18–25 age group category. The middle age groups had little difference among them; hence, the Chi-square established no relation between age group and topographical zones ($\chi^2 = 16.755$, $p = 0.334$).

Table 3.1: Social demographics presentation of respondents from four ecological zones of Lesotho.

Factor	Category	Highlands	Foothills	Lowlands	SRV	Totals	Statistics
				N (Means %)			
Gender	Female	7 (3.3)	11 (5.2)	23 (10.8)	6 (2.8)	47 (22.2)	χ^2 =4.753
	Male	40 (18.9)	40 (18.9)	56 (26.4)	29 (13.7)	165 (77.8)	p= 0.243
Age	18-25	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	χ^2 =16.755
	26-35	5 (2.4)	6 (2.8)	12 (5.7)	2 (0.9)	25 (11.8)	p=0.334
	36-45	15 (7.1)	10 (4.7)	17 (8.0)	4 (1.9)	46 (21.7)	
	46-55	9 (4.2)	11 (5.2)	13 (6.1)	6 (2.8)	39 (18.4)	
	56-65	8 (3.8)	14 (6.6)	19 (9.0)	7 (3.3)	48 (22.6)	
	66+	10 (4.7)	10 (4.7)	17 (8.0)	16 (7.5)	53 (25.0)	
Education	NFE	6 (2.8)	9 (4.2)	17 (8.0)	5 (2.4)	37 (17.5)	χ^2 =14.576
	Primary	28 (13.2)	24 (11.3)	35 (16.5)	15 (7.1)	102 (48.1)	p=0.265
	Secondary	6 (2.8)	9 (4.2)	17 (8.0)	4 (1.9)	36 (17.0)	
	High School	5 (2.4)	2 (0.9)	5 (2.4)	6 (2.8)	18 (8.5)	
	Tertiary	2 (0.9)	7 (3.3)	5 (2.4)	5 (2.4)	19 (9.0)	
Farming	< 5 years	1 (0.5)	5 (2.4)	10 (4.7)	3 (1.4)	19 (9.0)	χ^2 =11.735
Experience	6-10 years	3 (1.4)	13 (6.1)	13 (6.1)	6 (2.8)	35 (16.5)	p=0.068
	>10 years	43 (20.3)	33 (15.6)	56 (26.4)	26 (12.3)	158 (74.5)	
Total		**47 (22.2%)**	**51 (24.1%)**	**79 (37.3%)**	**35 (16.5%)**	**212 (100%)**	

N = actual frequency, SRV= Senqu River Valley, NFE= No formal Education

The respondents with primary and nonformal education recorded 48.1% and 17.5%, respectively. The primary education level was more pronounced in the Highlands with 59.6%. However, the Chi-square test did not establish any significant association (χ^2 = 414.576, df = 12, and p = 0.265) between the level of education and ecological zones at a 5% confidence interval. The majority of sheep farmers (n = 158, 74.5%) had an experience of more than 10 years, demonstrating most farmers have been rearing Merino for a long time. A chi-square analysis between farmer's age and years of experience per ecological zone was carried out and it revealed a significant association (χ^2 = 24.594, df = 8, and p = 0.002) existed only in the Foothills. The Chi-square analysis between gender and level of education revealed a significant association (χ^2 = 16.006, df = 4, and p = 0.003), in which males were more educated.

3.3.2. Farm management practices

The Merino sheep per farm were the highest in Highlands (104.080 ± 14.902) and the post hoc analysis also confirmed Highlands was significantly different from the other zones (Figure 3.2). The Lowlands registered the least number of sheep per farm (28.06 ± 3.636). Consequently, the ANOVA revealed the mean number of Merino sheep per farm was significantly ($p < 0.05$) different between the ecological zones in the study area.

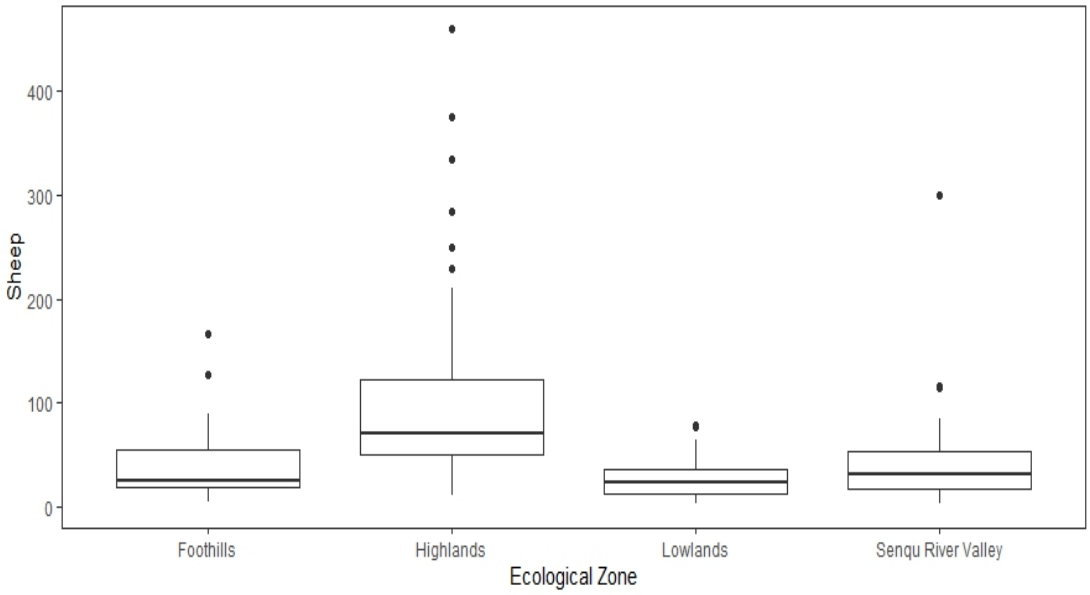

Figure 3.2: The boxplot graphical representation showing the mean number of Merino sheep distribution per farm in different ecological zones of Lesotho

The respondents also kept other species of animals on the same farm, including horses, donkeys, goats, cattle, and pigs. Table 3.2. show the mean number of animal species kept by sheep farmers across the four ecological zones of Lesotho. Goats were the second most abundant animal species after sheep, with an overall mean of 28.20 ± 2.549. The present study confirms sheep as the predominant livestock species reared in Lesotho. A two-tailed Pearson correlation analysis was established between farms rearing sheep and goats simultaneously and the result revealed a statistically significant and moderately positive correlation (R = 0.670, p = 0.01).

Table 3.2: Composition of other farm animal species owned by Merino sheep farmers in Lesotho

Animal species	**Highlands**	**Foothills**	**Lowlands**	**SRV**	**Mean total**
		Number, Mean (%)			
Horses	18 (1.94)	27 (4.59)	17 (1.41)	13 (2.85)	75 (2.93)
Donkey	15 (2.53)	27 (3.26)	35 (2.09)	14 (3.36)	91 (2.70)
Goats	31 (24.42)	39 (40.18)	17 (15.18)	14 (19.00)	101 (28.20)
Cattle	41 (7.07)	38 (10.92)	57 (6.35)	26 (6.46)	162 (7.62)
Pigs	6 (2.83)	8 (2.63)	17 (3.59)	1 (1.00)	32 (3.13)

SRV= Senqu River Valley

Table 3.3 shows some aspects of range management and feeding regimes for sheep in their respective ecological zones. Most of the respondents (72%) reported having a functional range management system in their areas. Three and two pasture divisions were the most common in different ecological zones, registering 28.4% and 21.8%, respectively. The maximum number of pasture divisions recorded was six. There was no correlation between the number of pasture divisions and the ecological zones. Transhumance in Lesotho is a seasonal pasture rotation system in which animals are sent to the mountainous animal posts during the summer. Farmers practicing transhumanism were marginally above half of all respondents (51.7%). Transhumance was practiced predominantly in the Highlands, as confirmed by 22.9% of the respondents. Foothills and SRV followed as second and third, with 16.1% and 12.8% overall, respectively. The dominance of the Highlands was further revealed by the overwhelming majority of respondents (93.6%) from this ecological zone who practiced transhumance. The association between transhumance practice and ecological

zones was assessed using the Chi-square and revealed a strong, statistically significant relationship (χ^2 = 149.481, df = 6, and p = 0.000). The Lowlands were the least likely (1.9%) to send their animals into the mountain animal posts. Some farmers kept their sheep on mountainous animal posts throughout the year but declined during the winter season (Figure 3.4). Eight months (October to May) showed that more than 65% of sheep farmers moved their flocks to remote animal post areas in the mountains. Farmers narrated different factors influencing their behaviour, one of which is that lambing ewes are brought to nearby village pastures for closer management and then sent back to highland animal posts after weaning. Furthermore, farmers lamented that the pastures around residential zones were relatively small and usually overstocked during the winter months; hence reluctance to leave the mountainous posts. July marked the lowest proportion of animals in the mountain animal posts.

Figure 3.3 Graphical presentation shows farms practicing transhumance and period of months Merino sheep are kept in the Highlands animal posts. Three feeding regimes recorded for Merino sheep farming across different ecological zones were: communal grazing combined with feeding supplementation, 100% communal grazing, and intensive feeding. Figure 3.4 represents the different feeding regimes practiced in the four ecological zones. The survey revealed that 90% (n = 190) of farmers head their animals to the communal rangelands across the study area, with exclusive communal grazing at 22.7%, communal grazing, and food supplementation regime at 67.3%.

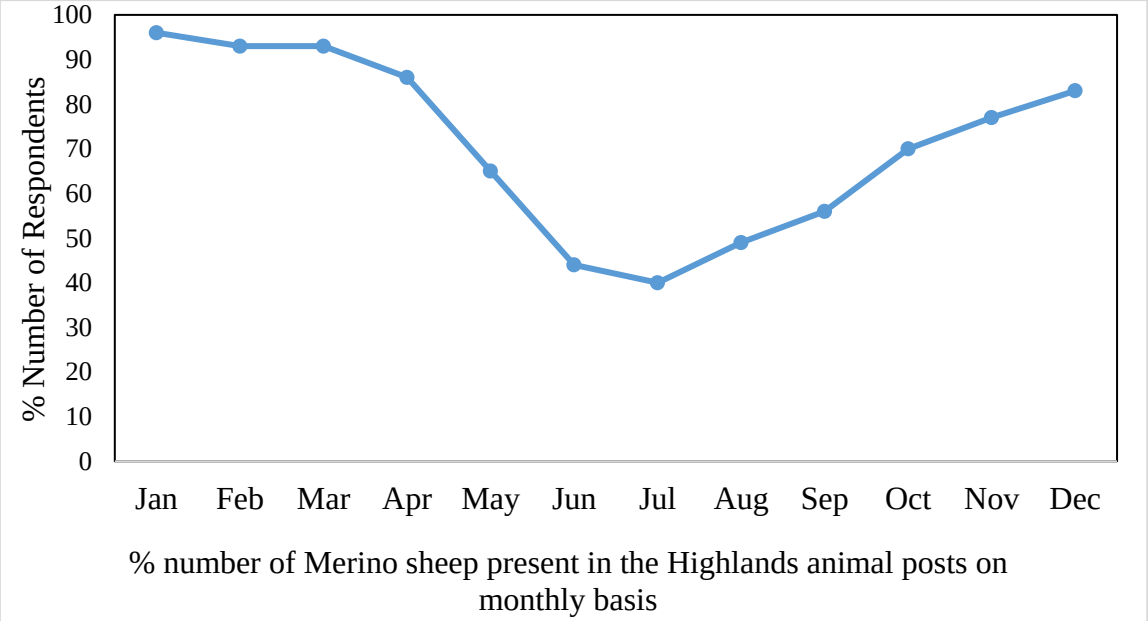

Figure 3.3: Graphical presentation showing transhumant farms and period in months Merino sheep spend in the Highlands' animal posts across Lesotho.

Table 3.3: Different management practices by farmers showing pasture management and feeding regimes by sheep farmers in Lesotho.

Management regime	Category	Ecological Zones					
		Highlands	**Foothills**	**Lowlands**	**SRV**	**Totals**	**Statistics**
Range management	No	8 (3.8)	13 (6.2)	19 (9.0)	10 (4.7)	50 (23.7)	χ^2 =7.783, df=6 p=0.254
	Yes	34 (16.1)	38 (18.0)	55 (26.1)	25 (11.8)	152 (72)	
Transhumance	No	3 (1.4)	11 (5.2)	72 (34.1)	8 (3.8)	94 (44.5)	χ^2=146.306, df=6 p=0.000
	Yes	44 (22.9)	34 (16.1)	4 (1.9)	27 (12.8)	109 (51.7)	
Pasture divisions	1	2 (0.9)	3 (1.4)	2 (10.9)	3 (1.4)	10 (4.7)	χ^2 =18.745, df=18 p=0.408
	2	9 (4.3)	10 (4.7)	18(8.5)	9 (4.3)	46 (21.8)	
	3	19 (9.0)	16 (7.6)	15 (7.1)	10 (4.7)	60 (28.4)	
	4	3 (1.4)	5 (2.4)	16 (7.6)	2 (0.9)	26 (12.3)	
	5	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	
	6	1 (0.5)	1 (0.5)	3 (1.4)	0 (0.0)	5 (2.4)	
Feeding Regime	CG +FS	32 (15.2)	34 (16.1)	53 (25.1)	23 (10.9)	142 (67.3)	χ^2 =2.851, df=9 p=0.90
	100% CG	12 (5.7)	13 (6.2)	16 (7.6)	7 (3.3)	48 (22.7)	
	IF	3 (1.4)	6 (2.3)	7 (2.8)	5 (1.9)	21 (9.9)	

SRV=Senqu River Valley, CG+FS = Communal crazing plus food supplementation, 100% CG = Communal grazing only, IF = Intensive Farming (zero grazing),

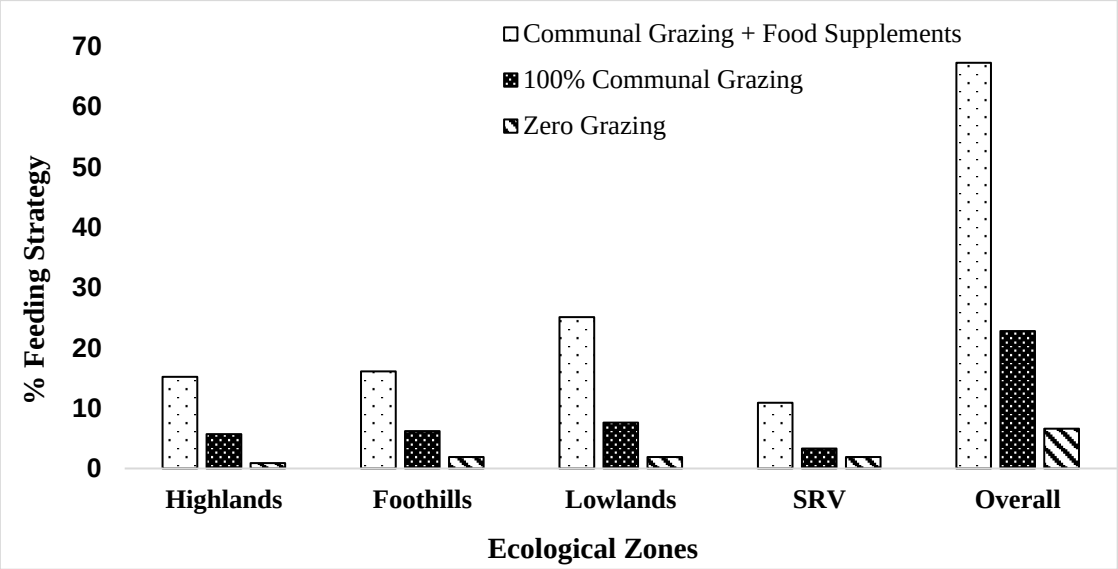

Figure 3.4: Animal feeding regimes practiced in Merino sheep farms in the four ecological zones of Lesotho.

3.3.3. Farmers' knowledge and perceptions of GIN infections in sheep

The survey on the sheep farmers' perceptions and knowledge and GINs' importance in their flocks revealed diverse viewpoints and responses (Table 3.4). The majority of farmers (84%) were familiar with the symptoms of helminthiasis. About a third of respondents (33.2%) acknowledged pastures were the source of GINs. Consequently, more than half of the Lowlands farmers reported pastures were the source of GIN infection in their animals. Figure 3.5. presents a graphical presentation of the perceived intensity and risk level of helminthiasis among Merino sheep farmers in the study area. Merino sheep farmers above 67% perceived the GIN infection to be serious and very serious across the study area.

Table 3.4: Farmers' knowledge and perceptions on symptoms of helminthiasis and source of infection.

Factors	Category	Highlands	Foothills	Lowlands	SRV	Totals	Statistics
Knowledge of Disease symptoms No. (%)							
Knowledge of helminthiasis signs	No	4 (1.9)	11 (5.2)	12 (5.7)	8 (2.4)	32 (15.2)	χ^2 =9.87, df=6
	Yes	43 (20.4)	42 (19.9)	64 (30.3)	29 (13.7)	178 (84.4)	p=0.239
Knowledge of the source of GIN infection No. (%)							
Parasites co-exist	No	41 (19.4)	43 (20.4)	46 (21.8)	24 (11.4)	154 (73.0)	χ^2 =15.242, df=6
	Yes	2(0.9)	1(0.5)	2(0.9)	1(0.5)	6(2.8)	p=0.18
From water	No	41(14.4)	40(19.6)	41(19.4)	24(11.4)	146 (69.2)	χ^2 =18.279, df=6
	Yes	2(0.9)	4(1.9)	7(3.3)	1(0.5)	14(6.6)	p=0.006
From other animals	No	43(21.8)	43(20.4)	46(21.8)	25(11.8)	157 (74.4)	χ^2 =4.432, df=6
	Yes	0 (0.0)	1(0.5)	2(0.9)	0 (0.0)	3(1.4)	p=0.008
From pastures	No	27 (12.8)	27 (12.8)	21 (10.0)	15 (7.1)	90 (42.7)	χ^2 =18.898, df=6
	Yes	16 (7.6)	17 (8.1)	27 (12.8)	10 (4.7)	70 (33.2)	p=0.004
From milk	No	34(16.1)	40(19.0)	43(20.4)	20(9.5)	137 (64.9)	χ^2 =18.279, df=6
	Yes	9(4.3)	4(1.4)	5(2.4)	5(2.4)	23 (10.9)	p=0.004
Do not know	No	23(10.9)	21(10.0)	34(16.1)	13(6.1)	91 (43.1)	χ^2 =20.101, df=6
	Yes	20 (9.5)	23 (10.9)	14(6.6)	12(5.7)	69 (32.7)	p=0.003

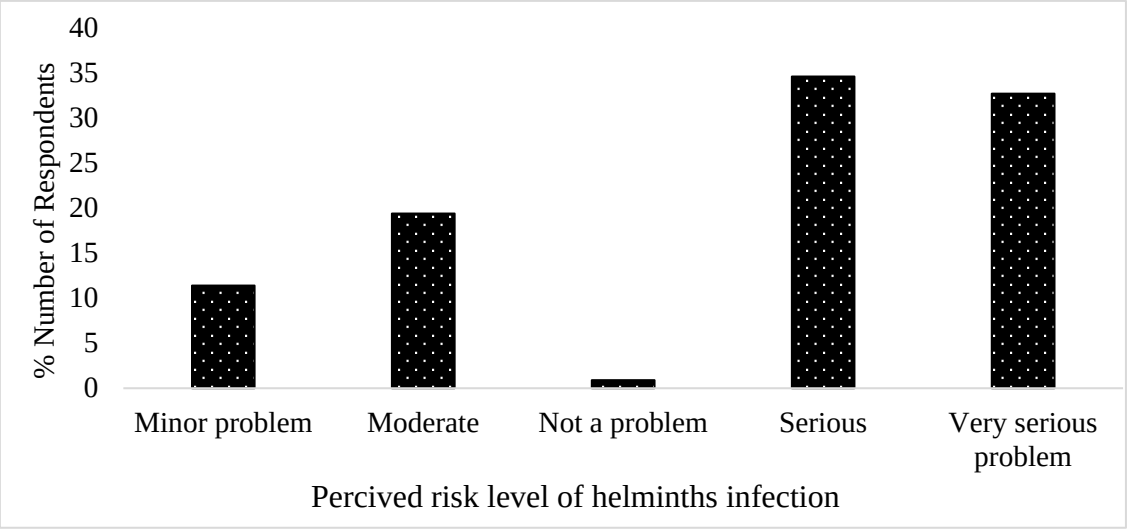

Figure 3.5: The percentage of farmers and their perceived significance level of helminthiasis infection in sheep

Figure 3.6 represents the percentage of respondents' knowledge of GIPs infecting sheep in the study area. Tapeworms and roundworms were the most commonly known GINs among farmers, with 66.8% and 55.9%, respectively. Respondents said tapeworms were detected more in lambs than in the adult sheep. 22.7% of the respondents did not know of any specific GINs infecting their animals, except that they cause devastating diseases. Apart from 33.2% of farmers who said animals were infected while they graze in the pastures, 32.7% did not know where GIN came from. A significant (χ^2 = 24.570, df=8 and p = 0.002) association was established by running the Pearson chi-square test of independence between knowledge of transmission and farmers' level of education.

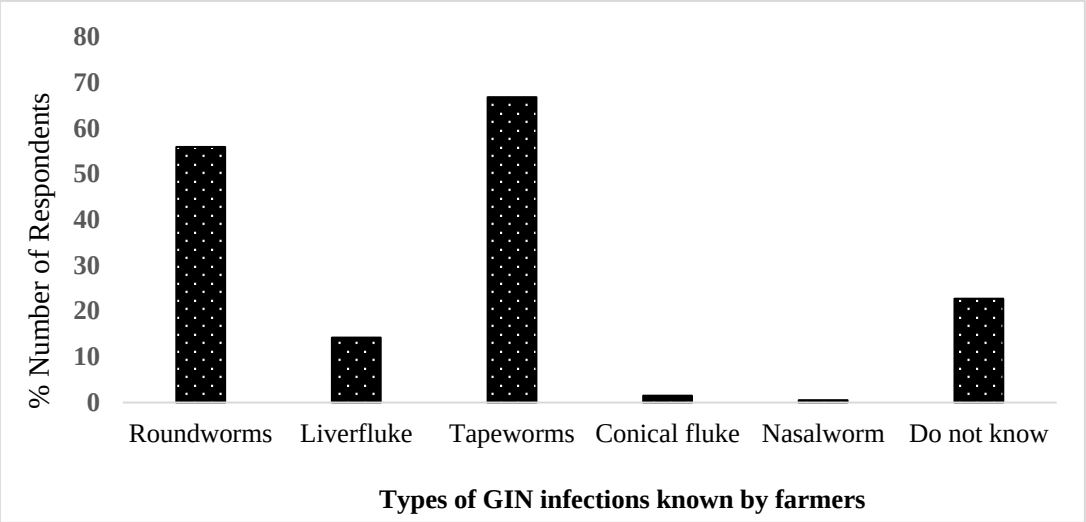

Figure 3.6: Percentage of farmers' knowledge of types of GIPs infecting sheep

3.3.4. Gastrointestinal Nematode control and Drug use among sheep farmers

The study recorded that a greater majority of farmers (98.1%) administered commercial anthelmintic drugs to their flocks. Table 3.5 represents farmers' responses on drug purchase, management, and administration in the study area. Respondents in the study got their drugs mostly from Lesotho drug outlets or farmer cooperatives (52.6%) and from South Africa (43%). The Lowlands farmers mainly bought drugs from local veterinary drug outlets (26.5%). A total of 61.6% of farmers spend more than M1000 (the equivalent of USD 60). The Pearson Chi-square test between the drug cost per farmer and ecological zones revealed a positive association (χ^2 = 30.079, df = 18, and p = 0.037) at 95% confidence. How farmers stored their anthelmintic drugs was also significantly different (χ^2 = 36.901, df = 9, p = 0.000), with those stored in an appropriate place and those that put them randomly in their living houses scoring 43.6 and 37.9%, respectively. However, approximately 66.0% of farmers from the Foothills reported the requisite etiquette for storing their drugs. In total, 69.7% of Lesotho Merino sheep farmers reported alternating anthelmintic drug brands on their animals yearly. The alternation of drug brands by farmers was assessed on a Pearson Chi-square test against ecological zones, and a significant relationship was established (χ^2 =16.452, df = 6, and p = 0.012). A majority of farmers (76.8%) administering drugs by themselves indicated they might always administer a rough estimation of drugs to their flocks. Among Ecological zones, 86.8% of farmers in the Foothills administered an

estimation of the drug. The Pearson Chi-square test revealed an association between drug administration regimes and ecological zones ($\chi^2 = 26.262$, df = 15, and p = 0.035).

Table 3.5: Details of drug purchase, management, and administration by Merino sheep farmers in four ecological zones of Lesotho

Category	Highlands	Foothills	Lowlands	SRV	Totals	Statistics
Sources of drugs						
Farmers' associations	5 (2.4)	8 (3.8)	3(1.4)	2(0.9)	18(8.5)	χ^2=7.865, df=6, p=0.248
NGO/Projects	1 (0.5)	1(0.5)	0(0.0)	0(0.0)	2(0.9)	χ^2=4.644, df=6, p=0.590
South African Coops	31(14.7)	34(16.1)	17(8.1)	10(4.7)	92(43.6)	χ^2=37.205, df=6, p=0.000
Vet clinics	5(2.4)	12(5.7)	7(3.3)	8(3.8)	32(15.2)	χ^2=9.238, df=6, p=0.161
Drug outlets-Lesotho	24(11.4)	14(6.6)	56(26.5)	17(8.1)	111(52.6)	χ^2=31.889, df=6, p=0.000
Extension officers	0	8(3.8)	6(2.8)	4(1.9)	18(8.5)	χ^2=10.210, df=6, p=0.116
Annual drug expenditure, N, (%)						
Other	1 (0.5)	4 (1.9)	3 (1.4)	4 (1.9)	12 (5.7)	χ^2=30.079, df=18, p=0.037
<M500	1 (0.5)	8 (3.8)	12 (5.7)	4 (1.9)	25 (11.8)	
>M1000	35 (16.6)	29 13.7)	47 (22.3)	19 (9.0)	130 (61.6)	
M500 to M1000	10 (4.7)	12 (5.7)	10 (4.7)	6 (2.8)	38 (18.0)	
Don't know	0 (0.0)	0 (0.0)	3 (1.4)	2 (0.9)	2 (0.9)	
Don't buy anthelmintic	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	
Drug storage N (% within ecological zones) (% Total)						
Other	0 (0.0) (0.0)	1 (1.9) (0.5)	4 (5.3) (1.9)	2 (5.7) (0.9)	7 (3.3)	χ^2= 36.901, df = 9, p= 0.000
Anyhow	21 (44.7) (10.0)	12 (22.6) (5.7)	37 (48.7) (17.5)	10 (28.6) (4.7)	80 (37.9)	
Conducive Place	25 (53.2) (11.8)	35 (66.0) (16.6)	20 (26.3) (9.5)	12 (34.3) (5.7)	92 (43.6)	
Don't Store Drugs	1 (2.1) (0.5)	5 (9.4) (2.4)	15 (19.7) (7.1)	11 (31.4) (5.2)	32 (15.2)	
Change of drug brand N (%)						
NO	11 (5.2)	11 (5.2)	32 (15.2)	6 (2.8)	60 (28.4)	χ^2 =18.279, df=6, p=0.012
YES	36 (17.1)	42 (19.9)	42 (19.9)	27 (12.8)	147 (69.7)	
Actual application of anthelmintic drugs N, (% Within EC) (% Total)						
Other	1 (2.1) (0.5)	0 (0.0) (0.0)	6 (7.9) (2.8)	2 (5.7) (0.9)	9 (4.3)	χ^2 =26.262, df=15, p=0.035
Extension officers	4 (8.5) (1.9)	2 (3.8) (0.9)	14 (18.4) (6.6)	1 (2.9) (0.5)	21 (10.0)	
Fellow Farmer	2 (4.3) (0.9)	2 (3.8) (0.9)	2 (2.6) (0.9)	1 (2.9) (0.5)	7 (3.3)	
Give rough estimation	38 (80.9) (18.0)	46 (86.8) (21.8)	50 (65.8) (23.7)	28 (80.0) (13.3)	162 (76.8)	
Manufacturer's dose	0 (0.0) (0.0)	3 (5.7) (1.4)	0 (0.0) (0.0)	1 (2.9) (0.5)	4 (1.9)	

Retail Personnel	2 (4.3) (0.9)	0 (0.0) (0.0)	4 (5.3) (1.9)	2 (5.7) (0.9)	8 (3.8)	
Increase drug dosage before the recent 5-year cycle, N (%)						
Other	1 (0.5)	6 (2.8)	8 (3.8)	5 (2.4)	20 (9.5)	χ^2 =9.857
No	24 (11.4)	21 (10.0)	31 (14.7)	21 (10.0)	97 (46.0)	df=6
Yes	22 (10.4)	26 (12.3)	37 (17.5)	9 (4.3)	94 (44.5)	p=0.131
Use of herbal concoctions, N (%)						
Other	0 (0.0)	1 (0.5)	0 (0.0)	2 (0.9)	3 (1.4)	χ^2 =13.209
No	19 (9.0)	27 (12.8)	24(11.4)	17(8.1)	87(41.2)	df=6
Yes	28 (13.3)	25 (11.8)	52 (24.6)	16 (7.6)	121 (57.3)	p=0.040
Total	47(22.3)	53 (25.1)	76 (36.0)	35 (16.6)	211 (100.0)	

N = actual frequency,

Figure 3.7 presents the period in a year farmers observe the onset of symptomatic animals with helminthiasis and their timing in administering anthelmintic drugs. More than 51% of farmers in the study area reported seeing helminthiasis symptoms in their flocks during the four months of summer: November, December, January, and February. Consecutively, a similar period appeared to reveal more than 40% of farmers administering anthelmintic drugs on their farms. Alternatively, May, June, and July portrayed a low percentage for the onset of helminthiasis and timing for drug administration. The patterns of both the appearance of helminthiasis symptoms and anthelmintic application in Merino sheep generally demonstrated congruence (Figure 3.7**).** The results portray helminthiasis observed more during summer than winter months.

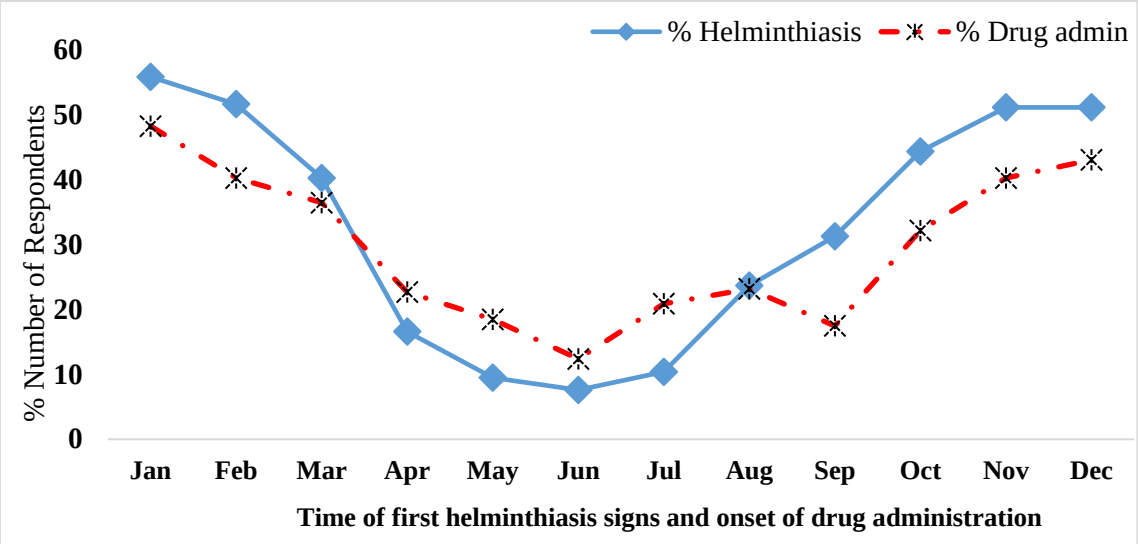

Figure 3.7: Farmer's perception of the onset of helminthiasis symptoms and timing for anthelmintic drug administration on Merino sheep in Lesotho.

Figure 3.8 is a graphical representation of the annual frequencies of drug administration before the last five-year period before the onset of the study and in the recent 12-month cycle of the interview survey (2023). The pattern revealed that 26.1% of respondents in the last five-year regime adopted "opportunistic" methods in administering anthelmintic drugs 'as and when they saw symptoms', a behaviour that declined remarkably with time. The norm was that the entire flock was treated, even if one or a few animals were diagnosed with helminthiasis symptoms. Apart from opportunistic treatment, farmers seemed to administer

drugs twice yearly which was second most prevalent. Alternatively, the latest most common annual drug administration frequencies depict three, twice, and quadruple in that order.

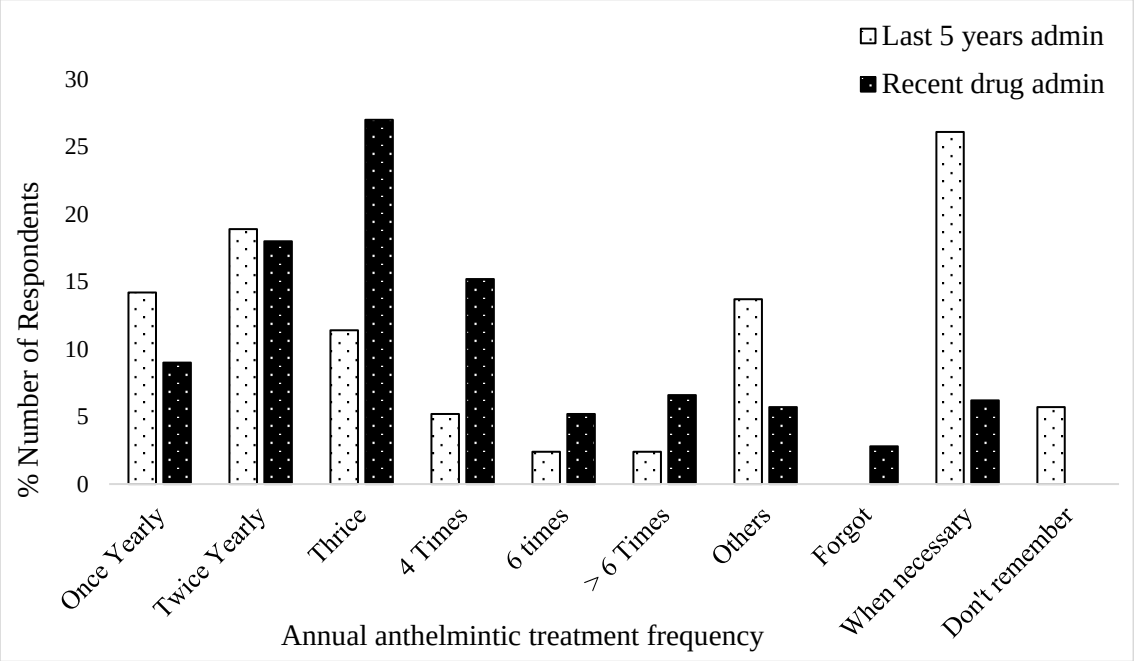

Figure 3.8: Annual anthelmintic frequency administration five-year prior and recent drug administrations.

Table 3.6 presents the data depicting the number of lambs and adult Merino sheep that showed helminthiasis symptoms and those that died in the six months before the date of the interview. Figure 3.9**.** represents the mean mortality of lambs and adult Merino sheep in the four ecological zones of Lesotho. The ANOVA and subsequent post hoc were used to establish the degree of the difference between symptomatic animals and animal mortality. The results revealed lamb mortality had a significant difference ($p < 0.05$), safe for adults. Mortality intensity between all age groups in the ecological zones. Were subjected to ANOVA The results also revealed that animal mortality was significantly different ($p < 0.05$) within the ecological zones, with the Highlands registering higher deaths than other ecological zones.

The results in the present study showed farmers received most of their guidance on the usage of drugs from extension personnel (42.7%) while Veterinary clinicians (38.9%) were second **(**Figure 3.10). Guidance from peer farmers was third while workshops were not common. The results portray that there was limited farmer training across the study area.

Table 3.6: Morbidity and mortality of different ages Merino sheep experienced in four ecological zones of Lesotho

Ecological Zones				
Highlands	Foothills	Lowlands	SRV	Total
		(Total No.) Mean		
Lambs infected with GIN				
(33) 25.45[a]	(37) 11.35[b]	(45) 6.51[b]	(19) 15.47[b]	(134) 13.78
Lambs died/farmer				
(40) 16.00[a]	(46) 5.43[b]	(61) 2.52[b]	(28) 3.64[b]	(175) 6.55
Adults infected with GIN				
(27) 13.89[a]	(33) 6.33[a]	(40) 8.68[a]	(14) 8.50[a]	(114) 9.21
Adult died/farmer				
(34) 2.06[a]	(41) 1.02[b]	(54) 1.54[b]	(23) 0.35[b]	(152) 1.34

Values with ([a,b]) are not significantly different (p = 0.05)

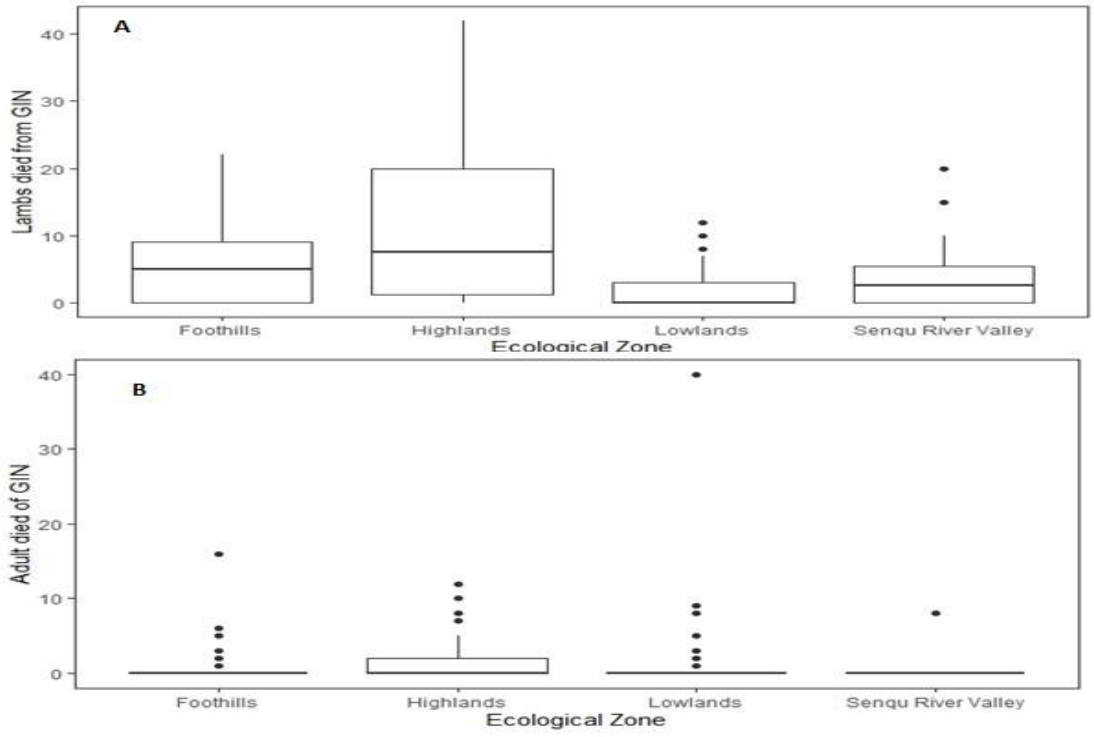

Figure 3.9: Boxplot presentation of mean number of lambs (A) and adult sheep (B) mortality due to GIN from different ecological zones of Lesotho.

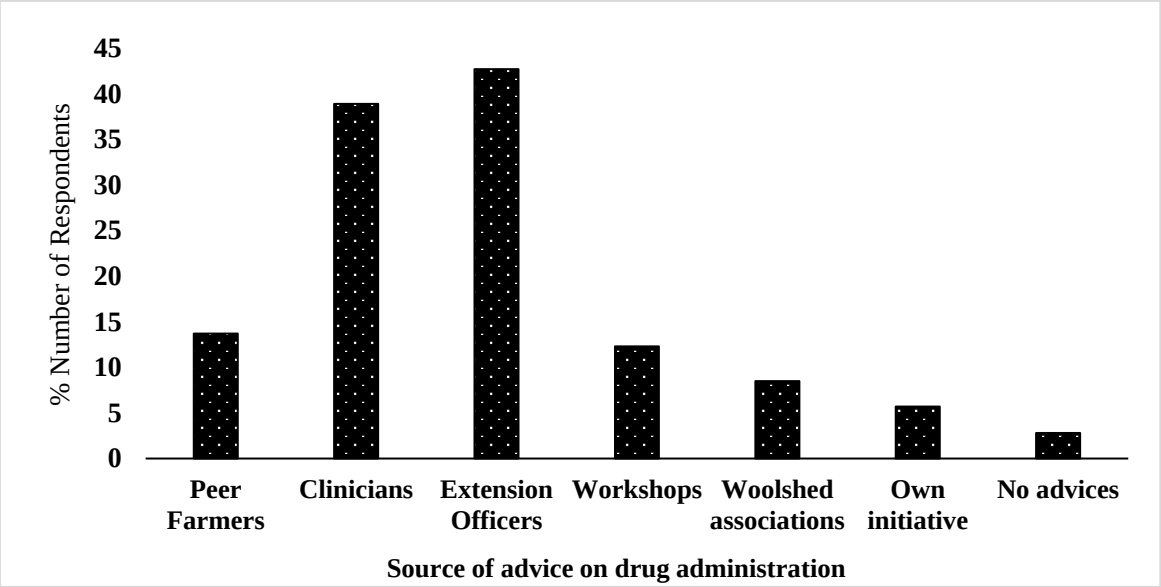

Figure 3.10: Graphical presentation of farmers' sources of advice on anthelmintic drugs administration.

3.3.5. Ethnoveterinary and Indigenous treatment against gastrointestinal nematodes of sheep in four ecological zones of Lesotho

There was an extensive use of herbal and indigenous remedies among farmers (57.3%), and that also resulted in a significant (χ^2 = 13.209, df = 6, and p = 0.040) relationship to ecological zones on Pearson Chi-square analysis. Table 3.7 presents the 11 most common plants farmers use in treating gastrointestinal ailments in sheep in the study area. Farmers used *Rumex lanceolatus, Phytolacca heptandra, Pelargonium sidoides, Zantedeschia albomacula,* and *Dicerocaryum ericapum* mainly for anthelmintic purposes. *Dicoma anomala, Zantedeschia albmaculata, Bulbine narcissifolia, Aloiampelos striatula, Rhamnus prinoides* and *Elephatorrhiza elephatina* were used to treat anaplasmosis. To alleviate the symptoms associated with diarrhoea farmers used *Eriocephalus tenuifolus* and *Pelargonium sidoides.* Disease conditions such as pulpy kidney and conical fluke were also mentioned as being treated by *Elephatorrhiza elephatina* and *Eriocephalus tenuifolus.* Some sheep farmers in Lesotho also used other indigenous methods apart from herbs. Farmers specified that the herbal remedies achieved the intended treatment objectives.

Table 3.7: Common plants used to treat gastrointestinal infections and other ailments of Merino sheep in the four ecological zones of Lesotho

Scientific name	Family Name	Local Sesotho Name	Ethnoveterinary Use
Zantedeschia albmaculata	Araceae	Mohalalitoe	Helminthiasis, conical fluke, Anaplasmosis
Aloiampelos striatula	Asphodelaceae	Mohalakane	Anaplasmosis
Dicoma anomala	Asteraceae	Hloenya	Anaplasmosis, Pulpy kidney
Bulbine narcissifolia	Asphodeloideae	Khomo-ea-balisa	Anaplasmosis
Rhamnus prinoides	Rhamnaceae	Mofifi	Anaplasmosis
Phytolacca heptandra	Phytolaccaceae	Monatja	Conical fluke and helminthiasis
Eriocephalus tenuifolus	Asteraceae	Sehalahala-sa-matlaka	Pulpy kidney/ enterotoxaemia, Diarrhoea,
Pelargonium sidoides	Geraniaceae	Khoara	Diarrhoea
Elephatorrhiza elephatina	Fabaceae	Mositsane	Conical fluke/ Pulpy kidney/anaplasmosis
Dicerocaryum ericapum	Pedaliaceae	Sesepa-sa-linoha	Helminthiasis, Pulpy kidney
Rumex lanceolatus	Polygonaceae	Khamane	Helminthiasis

3.4 Discussion

The success of the small ruminant industry worldwide depends on sound management systems that include anthropogenic-related factors, diseases, and parasite control. The Merino sheep rearing in Lesotho has existed for more than a century, and though challenges are encountered there is still room for improvement (Hunter, 1987). To achieve and maintain profitable margins in the livestock industry, knowledge of various aspects of the industry is essential. Livestock farming systems are categorized in size and management style, especially regarding the utilisation of food resources. Bernues *et al*. (2011) observed that the orientation of production, cultural roles, socio-economic dynamics, and aspects of farm profitability were so diverse and complex that there was no definite one-size-fits-all for its sustainability.

The present study revealed that the sheep industry in Lesotho is predominantly run by men aged between 45 and 66 years. The result is consistent with Moiloa *et al*., (2019) and Mahlehla *et al*., (2021) findings on similar demographics, in which the male dominance was

reported in small-stock farming in two districts of Lesotho. Livestock farming was historically and culturally deemed a man's job domain due to its intensive labour and long hours of herding. Transhumant farmers herded their flocks into remote and hard-to-reach mountainous areas in search of good pastures and to avoid grazing competition. In these areas, herders and their flock live in harsh and unbefitting conditions. According to cultural gender roles, this would not be appropriate for the female counterparts. It has consistently been reported that sheep farming in poor-resourced communities attracts more uneducated people, as reported in the present study. Adams and Ohene-Yankyera (2014) recorded that livestock farmers in Ghana were also predominantly uneducated.

Merino sheep are the dominant animal species reared mainly for their fleece, of which quantity and quality are the major trading attributes for the world markets. Lesotho Merino sheep farms hold small to medium-sized numbers (1-50 and 50-250) of animals (Shivakumara and Kiran, 2019). Consequently, the present study revealed that livestock farming in the four ecological zones of Lesotho exhibited a characteristic of an extensive farming system. This is shown by the predominant utilization of communal rangelands and low capital injection, hence the low-income production system. According to Shivakumara *et al*. (2016) and Shivakumara and Kiran (2019), this type of livestock farming can therefore be categorised as extensive production on account of low-income injection and all-year-round grazing on open communal pastures. Extensive farming systems are prone to several risks, including diseases and substandard animal welfare, which notably limit production gains. Turner (2013) cautioned where rangelands are the main source of animal nutrition, there is a need for judicious usage for reasonable and sustainable production. The present study revealed transhumance as a fundamental seasonal rotation regime for the Highlands, Foothills and SRV. Insignificant transhumance practice observed in the Lowlands in the current study was a culmination of a deliberate act of earlier government that was set to discourage overgrazing of the mountain rangelands (Turner, 2013). Alternatively, community-based range management initiatives were instituted under the chief's jurisdiction to encourage equitable pasture utilisation. The strategy introduced rotational grazing regimes that divided available communal pastures into several divisions for sustainability and to increase livestock value.

Though more capital injection is needed in intensive farming systems for feeds and labour, Shivakumara and Kiran, (2019) contended good prospects exist for increased production and profitability when management is done proficiently. Pfukenyi and Mukaritirwa (2013) reported that, apart from a sustainable nutrient supply, pasture rotation, and transhumance as mechanisms of pasture management may simultaneously be used as part of parasite control strategies. Alternatively, none of the respondents in the present study associated range management with either parasite prevalence, intensity, or control. Farmers perceived pastures as the animal's nutritional supply and pasture rotation may only be to give it a rest to recuperate. Their assessment of returning their animals to a pasture was only on the grass condition. Respondents engaged in intensive farming only constituted 9.9% in the current study. Kiran and Roy (2013) reported that though supplementation with concentrate mixes and minerals improved sheep production, its adoption among low-income farmers was sluggish. The intensive farmers' group in Lesotho's context ranged from intensive feeding with zero grazing to those with periodic grazing.

Seasonality in Lesotho is characterised by its relatively warm and humid summer months while winters are cold and dry (Phalatsi *et al*., 2022). There were mainly two and three pasture divisions in which animals rotated, periodically or seasonally in the case of transhumant flocks. Consequently, transhumance occurs predominantly during summer mainly from September to May. The quality and quantity of pastures improve relatively into summer when there is pronounced animal activity in the mountains, specifically for the Highlands, Foothills, and SRV. Migration back into the villages coincides with the decline of grasses on pastures in winter and harvesting crops like maize, which are by-products used for animal feeding. This may seem to be an alternative rather than supplementation due to the delicate state of the pastures during winter. A supplementation regime of 67.3% was a substantial advance compared to 22.7% of the absolute grazing regime which had no supplementation in the present study. According to Chandrawathini and Aini (2012), supplementation in livestock production has a propensity to improve the yield of both animal weight and fleece quantity and quality. Similarly, Kenfo *et al*. (2018) also observed that the paucity of animal feed supply was a major impediment to livestock production in Ethiopia.

The majority of respondents regarded GIN infection as a serious disease on their farms. Farmers' perceptions of the seriousness of GIN infection were in congruence with the studies

by Moiloa *et al*. (2019), Mahlehla *et al*. (2021), and Phalatsi *et al*. (2022), who recorded significantly higher GIN burdens in small ruminants in Lesotho. Relatively fewer farmers were aware that animals acquire GINs from the pastures (n = 90, 42.2%) while grazing than those who suggested infection from water (n = 146, 69.2%) and other animals (n = 157, 74,4%). This was lower than the findings by Mahlehla *et al*. (2021), who reported that 70% of farmers from Maseru and Quthing districts in Lesotho believed pastures were the source of GIN infection among Merino sheep. GINs were reported to be abundant and the most pathogenic GIPs of ruminants worldwide (Papadopoulos *et al*., 2003; Matebesi-Ranthimo, Jabba, *et al*., 2006). The two top GIPs known by farmers in the present study were roundworms and tapeworms. This result was similar to that reported by Ndlela *et al*. (2022) in Kwazulu Natal, South Africa. The only parasites observable with the naked eye were the tapeworm proglottids in faeces. This was also found by Tsotetsi and Mbati (2003), which reported that 87% of farmers in northeastern Free State, South Africa, could also easily identify proglottids from faeces, especially after deworming activity.

The overwhelming majority of farmers (98.1%) reported using commercial anthelmintic drugs. The current result revealed relatively higher anthelmintic use compared to that reported (80%) by Moiloa *et al*. (2020) and Mahlehla *et al*. (2021) for Maseru and Quthing Districts. Notwithstanding the increased use of commercial drugs among farmers in the study, a substantial number of respondents administered a rough estimation of drugs as opposed to a manufacturer's prescription. This may be attributed to the low education levels of farmers and the lack of requisite equipment for weighing animals for proper proportion of drug administration. Lindberg and Vatta (2006) emphasised that education was a basic requirement for empowering low-resource farmers with the needed skills for appropriate adherence to protocols that require substantial knowledge for improved management. Bath *et al*. (2016) also identified problems encountered by small ruminant farmers that could have been rectified by the provision of requisite education and training on aspects of livestock farming in the Eastern Cape province, South Africa. Lindberg and Vatta (2006) further reiterated that assessment of animals' health, adoption of requisite management skills, and subsequent epidemiological consequences were needed for effective management of daunting risks and to improve animal husbandry for increasing yields. Farmers buy drugs with instructions written in English, and this poses a serious language barrier and, consequently, elevated risk of non-adherence to the drug manufacturer's protocol. Several

factors agree concerning drug management and administration among farmers. Clinicians and extension officers were the two main guides on drug administration for farmers. Consequently, to avoid inappropriate usage of drugs for optimum parasite control and AR development, Ihler (2010) suggested veterinarians should solely be issuing drug prescriptions. About 77% of farmers who increased dosing frequency over five years cited reoccurrence of symptoms as the main motive.

A low percentage of farmers (1.9%) could read and independently administer the recommended drug dose. Most farmers did not weigh their animals and relied on administering a rough estimation of animal weight and a corresponding drug dose to their flocks. Indiscriminate drug administration was common among respondents and this could be one of the factors influencing AR development among GIN populations. The inconsistencies and erroneous drug applications are therefore an assumed cause of AR development in Lesotho GIN populations. Moreover, incorrect drug storage was also common and portrayed farmers' ignorance and risk of compromised drug efficacy and also a contributing factor to the advanced AR in GINs (Vande Velde *et al*., 2018). Melville *et al*. (2016) reported that the increased frequency of anthelmintic administration for control of GIN leads to the inevitable development of resistance within the parasite population. The two groups of GIN exist, one in the host and exposed to drug treatment and the other in the environment unexposed to anthelmintic drugs, usually referred to as refugia. The rate of AR development is dependent on the relative number of parasites between the parasitic phase and the refugia populations. Consequently, Melville *et al*. (2016) also hinted that the practice of repeated whole flock treatment also reduced the diversity of refugia, leading to relatively accelerated AR development. On the other hand, knowledge of resistance to particular drugs in circulation may assist in informing strategic drug rotation, which is prudent for controlling the parasite population and eventually managing AR development. Miller *et al*. (2012) reported significant economic gains when lambs were treated with a drug combination in contrast with known benzimidazoles which recorded less than 50% efficacy against *Trichostrongylus* and *Teladosargia* species. These results demonstrated a need for extensive investigation before, during, and after the introduction of a new drug to track AR development and act accordingly. The high intensity and indiscriminate use of anthelmintic drugs are daunting AR challenges in Lesotho, exacerbated by the use of common grazing pastures and the transhumance system. There was inconsistency in the overall usage and

management of drugs within and among farmers across four ecological zones. The erroneous drug administration etiquettes in the present study are similar to those reported by Mphahlele *et al*. (2021), who also suggested that extensive and disorganised use of drugs owing to insufficient knowledge and a lack of proper supervision of drug administration may select for AR mutations. Moreover, Ihler (2010) and Mphahlele *et al*. (2021) reported that animal nutrition, pasture management, breeding helminth-resistant sheep, and the correct use of anthelmintic drugs were important factors for sustainable GIN control in small ruminants. Consequently, the level of business engagement is also an important contribution to the overall industrial output.

The inadequacies inherent to resource-poor communities as observed by Bresciani *et al*. (2017) reiterate the impracticability of sufficiently incorporating all important factors into animal production. The limited pasturelands in Lesotho, alluded to by Turner (2013) and the overarching dominant use of communal grazing are serious phenomena that have potential risks for the parasites spread and AR development among sympatric ruminants. Moiloa *et al*. (2020) and Mahlehla *et al*. (2021) mentioned disparities such as animal species, host movement, husbandry regimes and anthelmintic control strategies in small ruminant management in Maseru and Quthing districts in Lesotho. More farmers keep mixed animal species in their farms, which are all herded in the same communal pastures. The drug application regimes among farmers from the same area also differed owing to their financial disparities, education levels, availability of veterinary services and experience of farmers. Consequently, this may also contribute to the general farm management, parasite controls and risk of AR development.

Herbs have been historically used for both human and animal disease treatment across the globe (McGaw and Elloff, 2008). Overall, 57.3% of respondents used herbal remedies and indigenous methods to treat GINs and other closely related ailments in their sheep flocks across four ecological zones in Lesotho. Ndlela *et al*. (2022) reported that Indigenous knowledge was orally passed from one generation to the next among farmers' communities and families., McGaw *et al*. (2020), and Motsoari *et al*. (2022) mentioned similar herbs were used, even though the details of the target ailments may differ slightly from the present study. McGaw and Eloff (2008) reported the use of *R. lanceolatus, Z. albomacula*, and *P. sidoides* against helminth symptoms. Dicoma anomala was used to treat gallsickness and wounds in

small ruminant animals (McGaw *et al*., 2020; Motsoari *et al*., 2022). *Rhamnus prinoides* was reported to treat anaplasmosis, while Yirga *et al*. (2012) reported a similar plant was used to alleviate bloat in ruminants. Some herbal remedies used by farmers, such as *Rumex lanceolatus* and *B. narcissifolia*, were used for sexually transmitted diseases (Kose *et al*., 2015). *Elephantorrhiza elephatina* was used to treat several ailments in humans, such as heartwater, black-quarter disease, and syphilis (McGaw and Elloff, 2008; Kose *et al*., 2015). Farmers reported that the herbs alleviate the symptoms of intended ailments hence their continued administration on their flocks.

3.5 Conclusion

The present-day study revealed that Merino sheep farming is a male-dominated venture in Lesotho. Farmers in the Highlands are significantly engaged in the transhumance system of seasonal pasture rotation. Moreover, Highlands hold significantly higher numbers of Merino sheep per farmer than other ecological zones. Merino sheep farmers predominantly use communal pasture. There was overwhelming use of commercial anthelmintic drugs to treat GINs among Merino sheep farms. Benzimidazole, niclosamide, levamisole, and ivermectin drug groups were the four most common drugs used in Lesotho. The study also revealed summer experienced more parasite and treatment activities. The Highlands exhibited Merino sheep in mountain animals' posts throughout the year although with a significant decline during winter as a result of migration to pastures close to village settlements. There has been an increase in the frequency of drug administration in sheep in the last five-year period 2021/22. The Highlands experienced significantly ($p < 0.05$) higher lamb mortality than other ecological zones. Slightly above half of Merino sheep farmers use herbal and indigenous treatments against GINs and other ailments in their flocks. Overall, the alternative hypothesis (H_1) that farmers from four ecological zones of Lesotho have significantly contrasting livestock management and GIN control strategies was supported and therefore accepted.

CHAPTER 4

Cross-sectional survey of occurrence, abundance, and prevalence of gastrointestinal parasites of Merino sheep in Lesotho

4.1. Introduction

Small ruminant production is one of the major socio-economic activities in rural and resource-poor areas of Lesotho. Merino sheep in particular have been a significant contributor to the country's GDP through wool sales for more than a century (Hunter, 1987). Livestock production in Lesotho relies predominantly on communal grazing. The communal grazing regimes pose serious challenges regarding the concept of the tragedy of the commons and the overall management and control of animal diseases in rural communities. Lesotho farmers practice transhumance as an integral part of the communal grazing system (Mbata, 2001). Tsotetsi and Mbati (2003) noted that communal grazing favoured more distribution and increased infections among animals in overlapping pastures. Gastrointestinal parasite (GIP) infection is one of the leading health threats against a successful and productive small ruminant industry all over the world (Asif *et al.*, 2008; Cai and Bai, 2009). GIPs of importance in sheep and goats belong to three major groups: nematodes (roundworms), trematodes (flukes), and cestodes (tapeworms) (Moiloa *et al.*, 2019; Matsepe *et al.*, 2021; Mahlehla *et al.*, 2021). GIPs directly cause significant economic losses through animal illness, death, premature slaughter, and the condemnation of carcasses by abattoirs (Over *et al.*, 1992; Wang *et al.*, 2006). Indirect losses manifest in low feed conversion ratios, which result in thriftiness, weight loss, low milk production, poor fleece production, and anaemia (Richard *et al.*, 1954; Lebbie, 1994; Afridi *et al.*, 2007) Furthermore, control strategies increase losses, especially with emerging AR to broad-spectrum drugs. Regular diagnosis of livestock diseases is an important venture for maintaining a healthy flock by monitoring parasites for timely treatment and control. Inevitably, the differential diagnosis of *H. placei* parasitizing cattle and *H. contortus* infecting small ruminants poses a huge challenge due to their close similarities, especially where their hosts are sympatric (Zarlenga *et al.*, 2016). The magnitude and contribution of small ruminants to Lesotho's livelihood dynamics warrant a thoughtful commitment to

periodic epidemiological surveys to predetermine potential threats to the industry and curb imminent losses. The current initiative investigated the prevalence, diversity and intensity of gastrointestinal parasites in Merino sheep in the four ecological zones of Lesotho.

4.2. Materials and Methods

4.2.1. Experimental design

A stratified randomized cross-sectional study was carried out to evaluate the diversity, prevalence, and intensity of GIPs in Merino sheep in Lesotho. The study was conducted by collecting faecal samples from Merino sheep between December 2021 and January 2022. The study area was stratified into four ecological zones and samples were collected from 20 collection areas as explained in Chapter 3. The Raosoft sample size calculator was used to determine the minimum sample size at 95% confidence limit. Age-wise sampling was structured such that two farmers from the collection site provided 15 sheep, and five animals representing each of the three age categories: lambs (>1 year), yearlings (1 to 2 years), and adults (>2 years). In total, 602 faecal samples were collected from Merino sheep across four ecological zones in Lesotho. Faecal samples were collected per rectum using surgical gloves and put into sample bottles. Each bottle was assigned a unique identifier corresponding to the specific collection site. The sampled animals were predominantly grazed in communal rangelands governed under distinct chiefs' and/or range management areas.\ The faecal samples were then placed in cooler boxes and transported to the Central Veterinary Laboratory in Maseru for identification and quantification of GIPs' egg and oocyst for each animal. To determine the effect of sheep's management by age and disparities in GIP infection dynamics, different sheep age groups were considered during sampling.

The McMaster method of faecal egg count (FEC) was applied in the present study. GIP eggs and oocysts were identified under the microscope as per guides from Indre *et al.* (2010), Shorb (1939), and Soulsby (1982). Two grams of faeces were weighed, mixed with 58 ml of saturated sodium chloride solution (40%), and ogenized. The homogenate was then sieved through a tea strainer and filled in two chambers of a McMaster slide. The setup was left to stand for three minutes. Strongyle and *Monezia* spp. eggs and *Eimeria* spp. oocysts were counted within the two demarcated grids.

The faecal egg count was calculated as egg per gram of faeces (EPG) and oocyst per gram faeces (OPG) using the following formula:

EPG/OPG (number of eggs/oocysts) = (actual counts from two chambers) × 100

4.2.2. Coproculture preparation, and larval identification

The faecal samples were transported to the laboratory in airtight medical sample bottles, chilled in a cooler box, and then refrigerated at 4 °C. Five cultures of pooled faecal samples, each representing a distinct grazing area (collection site) from each ecological zone, were processed for coprocultures. Faecal samples and vermiculite were mixed in a 1:1 ratio, moistened, and an antifungal agent was introduced into the mixture to prevent fungal growth. The mixture was prepared in an apparatus of two different-sized glass containers, in which the inner container was filled up to three-quarters high and placed into the large jar. Water was then poured into the bigger glass jar for larval harvesting. Coprocultures were then incubated at 37 °C for up to six days, and hatched larvae were harvested. Figure 4.1 shows the method of faecal collection (from the rectum), parasites, and some class apparatus used for coproculture preparation.

Harvested L_3 from coprocultures were washed to remove excess debris. The L_3 was separated by sieving, washed several times in distilled water, and then suspended in phosphate-buffered saline (PBS). The larval suspension was identified by using morphological and morphometrical microscopy techniques. The method comprised the distinction of L_3 cranial extremities (head shape), the number of intestinal cells, measuring, and calculating the sheath tail extension (STE) value (´X´). A maximum of 100 L_3s were screened and counted, individually identified by the above-described features. L_3 was measured under a compound microscope equipped with an ocular micrometre. Morphometrical analysis of L_3 was done as per the protocol by van Wyk and Mayhem (2013). They recorded *H. contortus* larvae STE of 65–82 µm and *H. placei* measured about 80–119 µm. The result portrayed *H. contortus* as having relatively shorter STE and the ´X´value of STE was computed as 2.2–2.7 while *H. placei* was 2.7–4.0. The measures are

used as a standard for reference in larval identification. Figure 4.2 shows pictures and a diagrammatic presentation of the L_3 of *H. contortus*. Five morphometrically identified L_3s were pelleted at 14000 rpm for 10 minutes and suspended in 70% ethanol in 1.5-ml Eppendorf tubes for downstream analysis.

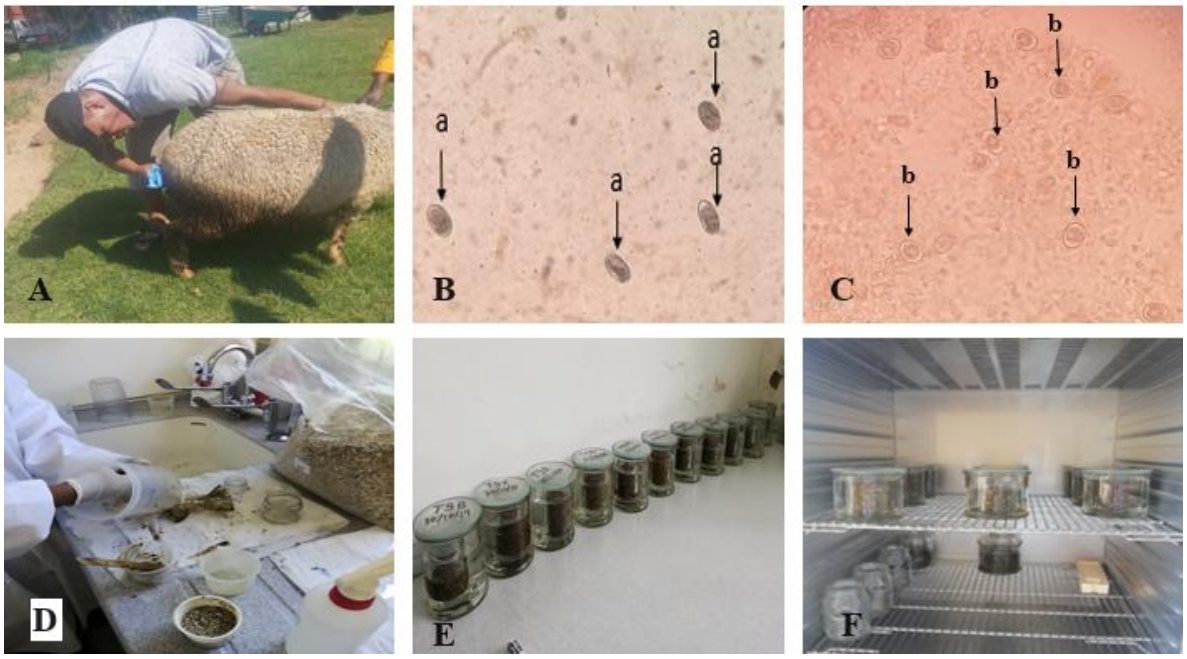

Figure 4.1: (A). Shows the researcher's collection of faecal samples directly from sheep's rectum using surgical gloves. (B). Isolated GIN eggs as seen from under the microscope (X100), shown by arrows and letter (a). (C). Microscope images of *Eimeria* species (X100), shown by arrows and letter (b). (D). Worker preparing a coproculture by mixing faecal matter with vermiculite. (E) Class apparatus used in coproculture in which eggs in the faecal matter were incubated and strongyle L_3 was eventually harvested. (F) an image of samples in the incubator.

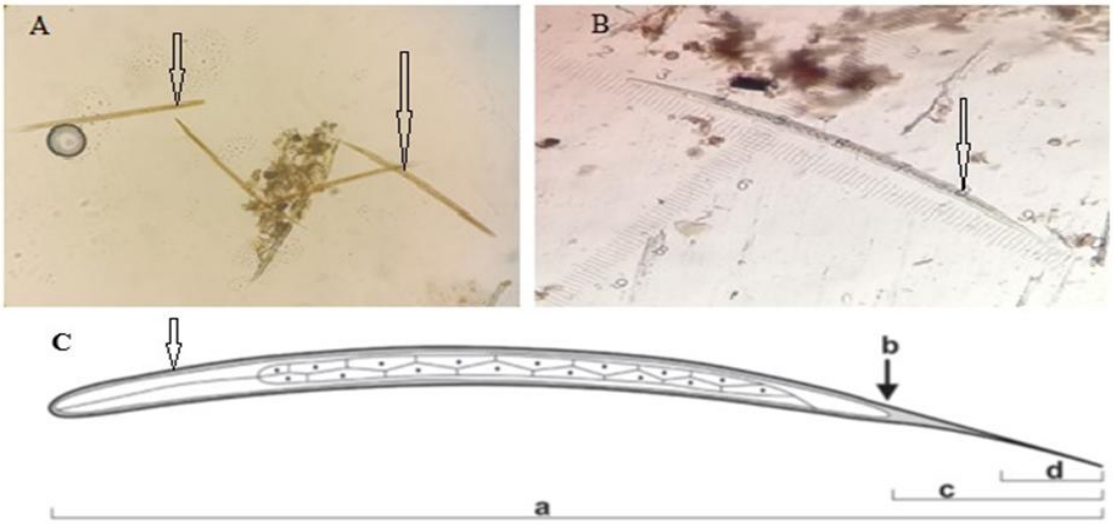

Figure 4.2: Pictorial presentations (A) and (B) portray the third-stage larvae from faecal sample (shown by arrows). C, the diagrammatic presentation of a nematode third stage larva showing different characteristic morphological features (a) total body length, (b) tip of larva tail, (c) sheath tail extension, and (d) the filament (adapted from van Wyk and Mayhew, 2013) diagram not to scale.

4.2.3. Data analysis

Descriptive statistics was used to organize and analyse numerical data. The SPSS 20.0 package was used for statistical analysis of gastrointestinal prevalence, burdens, and L_3 identification parameters. Analysis of variance (ANOVA) was used to analyze statistical differences between ecological zones and to test the dynamics of several GIPs (Girden, 1992). Univariate analysis of variance and the Tukey post-hoc test were used to differentiate between ecological zones.

4.3. Results

4.3.1. Prevalence and abundance of gastrointestinal parasites

Three types of parasites were identified from sheep faecal matter in the present study area that comprises Strongyles eggs, *Monezia* spp. eggs, and *Eimeria* spp., recording 64%, 18%, and 1.3% of overall prevalence rates, respectively. There was a significant difference ($p < 0.05$) in the GIPs recorded in the study on the prevalence among the ecological zones. Strongyles were the most prevalent and abundant of all GIPs identified in the present study.

Strongyles burdens were significantly ($p < 0.05$) different across the ecological zones, with the Highlands registering higher counts (1170.97 ± 113.134), followed by SRV (673.76 ± 75.651) (Table 4.1). *Monezia* spp. counts in the present study did not show any significant difference ($p > 0.05$) between ecological zones. The age-wise comparisons for all recorded GIPs did not show any significant difference for egg/oocyst counts or prevalence rates. The results showed a significant difference ($p < 0.05$) in the prevalence of strongyles and *Eimeria* spp. across Lesotho. Lambs had the highest infection rates for all GIPs, strongyles eggs, *Monezia* spp. eggs, and *Eimeria* spp. oocysts, with 658.42 ± 75.211, 295.052 ± 59.332 and 5.45 ± 4.505, respectively. The age-wise comparisons were not significant ($p > 0.05$) among all age groups between the ecological zones. Lambs also had the highest non-significant (65 ± 0.034, $p > 0.05$) prevalence, which deviated slightly relative to the other two group ages. There was no significant difference ($p > 0.05$) among *Eimeria* oocyst counts in the study area, even though the highest estimated marginal mean (446.39 ± 321.738) was recorded for the Foothills.

There was no significant ($p > 0.05$) difference between the strongyle burdens in Highlands and SRV. Figure 4.3 represents the boxplot of the mean strongyle burden in sheep across different ecological zones of Lesotho. Figure 4.4 represents the boxplot of the mean number of strongyle burdens by sheep age in different ecological zones of Lesotho.

Table 4.1: The intensity and prevalence of Strongyles, *Monezia* spp, and *Eimeria* spp presented as egg/oocysts per gram (epg/opg) of faeces per ecological zones and by age from sheep.

Parasite	EC	N	% PREV	EMM	SD.	SE
		Prevalence and Parasite intensity				
Strongyles	Highlands	141	89[b]	1170.97[b]	1259.809	113.134
	Foothills	124	62[a]	483.73[a]	989.172	76.775
	Lowlands	166	4[c]	183.63[c]	508.829	38.911
	SRV	171	72[a]	673.76[a]	898.304	75.651
	Total	**602**	**64**	**584.55**	**987.936**	**40.265**
Monezia spp	Highlands	141	0[a]	0.00[a]	0.000	0.000
	Foothills	124	4[a]	4.82[a]	26.531	2.059
	Lowlands	166	1[a]	0.58[a]	7.647	0.585
	SRV	171	1[a]	6.38[a]	75.794	6.383
	Total	**602**	**1.3**	**2.99**	**39.435**	**1.607**
Eimeria spp	Highlands	141	27[b]	45.97[a]	121.908	10.948
	Foothills	124	27[b]	446.39[a]	4145.310	321.738
	Lowlands	166	06[a]	7.06[a]	27.898	2.140
	SRV	171	12[a]	17.73[a]	56.421	4.752
	Total	**602**	**18**	**138.94**	**2183.075**	**89.049**
		Age-wise				
Strongyles	<1yrs	202	65[a]	658.42[a]	1068.944	75.211
	1-2 yrs	193	62[a]	495.85[a]	836.401	60.205
	>2 yrs	207	63[a]	595.17[a]	1033.317	71.820
	Total	**602**	**64**	**584.55**	**987.936**	**40.265**
Monezia spp	<1 yr	202	2[a]	5.45[a]	64.028	4.505
	1-2 yrs	193	1[a]	1.04[a]	10.153	.731
	>2 yrs	207	1[a]	2.42[a]	20.761	1.443
	Total	**602**	**1**	**2.99**	**39.435**	**1.607**
Eimeria spp	<1 yr	202	18[a]	295.05[a]	3685.804	259.332
	1-2 yrs	192	17[a]	91.67[a]	786.025	56.726
	>2 yrs	207	18[a]	30.43[a]	122.242	8.496
	Total	**601**	**18**	**138.94**	**2183.075**	**89.049**

EC = ecological zone, N = Number of sheep observed, Mean% Prev = Mean Percentag
Prevalence, EMM = Estimated Marginal Means, SD = Standard Deviation, SE = Standard Erro
Estimated marginal means with similar superscript [(a,b)] are not significantly different ($p \geq 0.05$

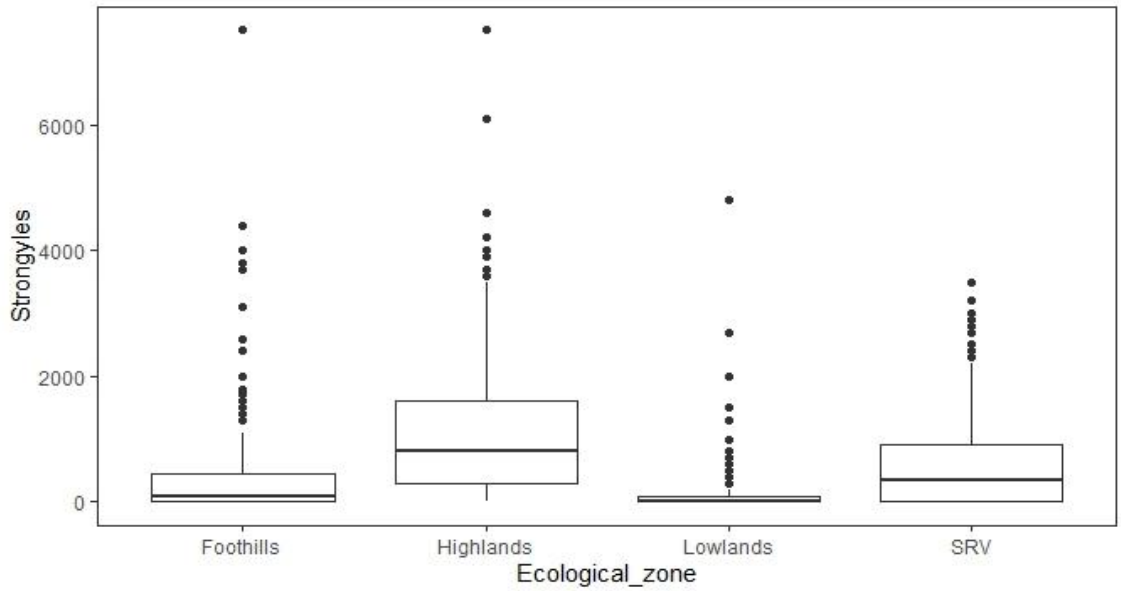

Figure 4.3. The boxplot demonstrates the graphical mean strongyles burden distribution in Merino sheep in Lesotho.

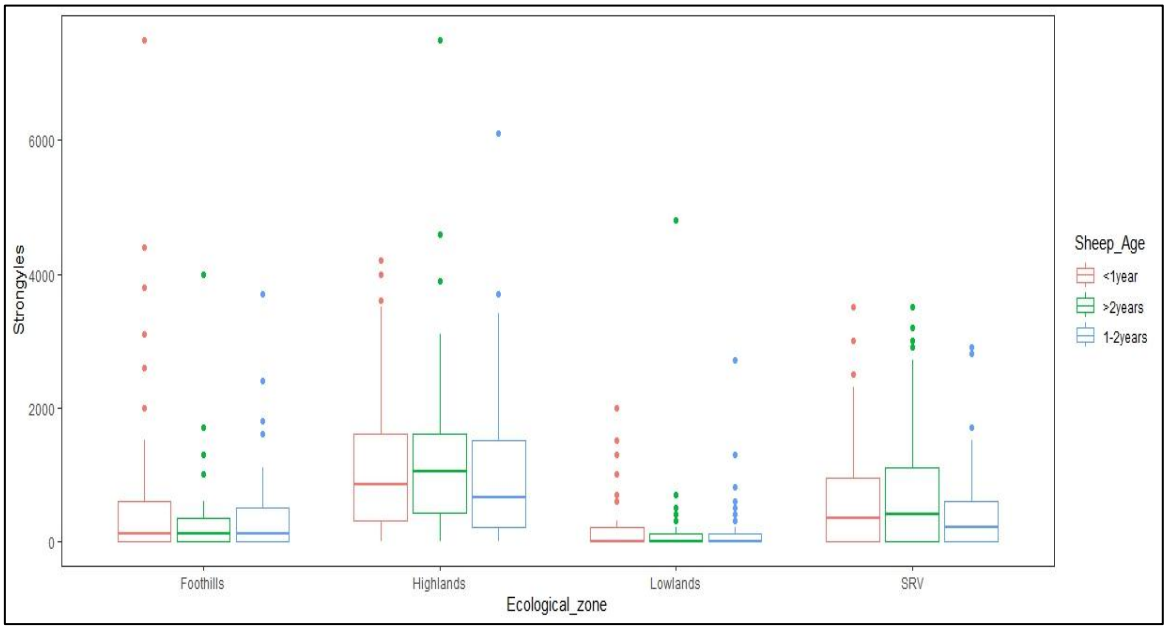

Figure 4.4. The boxplot depicting a graphical presentation of the mean number of strongyles burdens by sheep age from four ecological zones

4.3.2. Morphometrical identification of larval GINs

Only samples with up to 200 EPG were used for culturing to ascertain a positive result. Faecal samples from a similar collection site were pooled and prepared for coproculture.

Sheep pellets were crushed and mixed with vermiculite at a 1:1 ratio to provide good aeration for eggs. A two-jar method of culturing was used in which a smaller jar was first stuffed three-quarters high with faecal matter. The stuffed container was placed in a bigger water-filled jar. The coprocultures were then incubated for six days, and L_3s were water harvested in the outer jar. The L_3 was then processed as per van Wyk and Mayhew (2013) for morphological identification. The infective larvae possess a characteristically slender body with a sheath tail, a bullet-shaped cranial end, and 16 intestinal cells (van Wyk and Mayhew, 2013). The protocol makes the larvae relatively straight for ease of measuring the larvae and their sheath tail extension (STE). The preparation method retained the larval visible and translucent internal appearance for a clear distinction of target features. Larval suspensions were then analysed in McMaster counting chambers and identified under the microscope. A maximum of 100 larvae were considered for larval identification. Larval suspensions that had less than 100 individual larvae were all identified and counted. Five straight and positively identified L_3s from each collection site were selected for measurement using a calibrated ocular micrometer. The overall process of morphometrical analysis involved identifying and measuring the relative length of the STE, which starts from the tip of the larval tail towards the end. The shape of the cranial end ('head') region is used to differentiate L_3 for a more conclusive result. The tail also has a characteristic kinked profile along its length. All prepared coprocultures yielded strongyle L_3. Figure 4.5 represents three different aspects of L_3 morphology as seen under the compound microscope. All observed L_3 had a characteristic bullet-shaped cranial/head region and had a characteristic kinked filament. The overall length of the L_3 was 661.4444 ± 5.18223 mm while the sheath-tail extension (STE) measured 81.6944 ± 1.30472 mm (Table 4.2). The larvae were 100% *H. contortus* upon morphometrical identification as per van Wyk and Mayhew (2013).

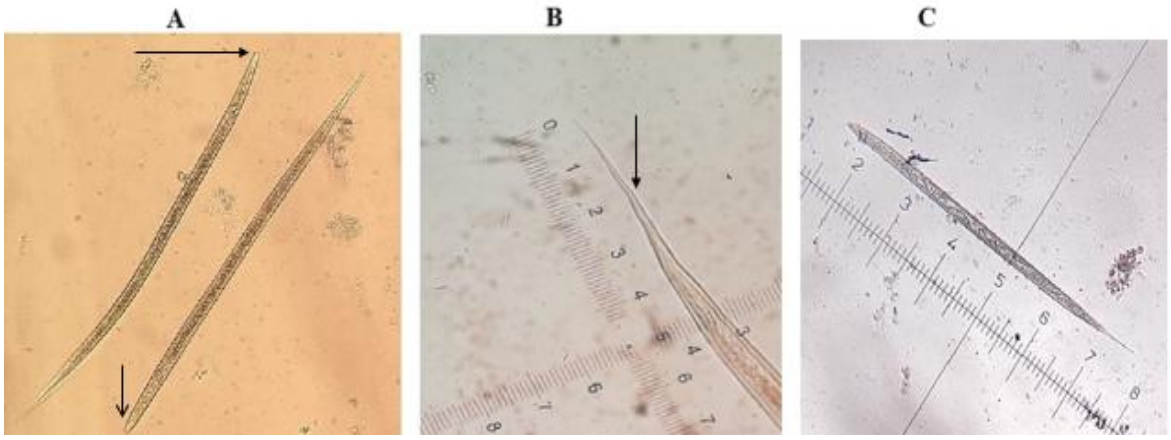

Figure 4.5: Pictures of *H. contortus* L_3 under the microscope with an ocular micrometer. (A), the bullet head cranial end (X100), (B), STE caudal end (X400). and (C), full body length (X100)

van Wyk and Mayhew's (2013) protocol on the identification of the L_3 of *H. contortus* of small ruminants STE calculation was set in the range 2.2–2.7 ´X´while the equivalent values for *H. placei* of cattle ranged from 2.7–4.0 ´X´. The present study revealed the average STE value of GIN in evaluation with ´X´=2.4752 ± 0.03953. Consequently, the GIN STE value recorded in the present study corroborated the result from van Wyk and Mayhew (2013) and identified and characterized as *H. contortus*.

Table 4.2: Measurements of different aspects of third-stage larvae of GINs of sheep from four ecological zones of Lesotho

Factors	EZ	N	Mean µm	Std. Dev	Std. Error
Total body Length	Highlands	25	668.8000[a]	58.33238	11.66648
	Foothills	25	635.2000[ab]	43.88622	8.77724
	Lowlands	25	667.2000[ab]	38.57028	7.71406
	SRV	15	683.3333[b]	42.20133	10.89634
	Total	**90**	**661.4444**	**49.16291**	**5.18223**
Length of STE	Highlands	25	80.0[a]	10.63113	2.12623
	Foothills	25	79.0[a]	8.80932	1.76186
	Lowlands	25	84.4[a]	17.82847	3.56569
	SRV	15	84.5[a]	7.91698	2.04416
	Total	**90**	**81.6944**	**12.37765**	**1.30472**
Value ´X´ of STE(C)	Highlands	25	2.4244[a]	0.32287	0.06457
	Foothills	25	2.3936[a]	0.26661	0.05332
	Lowlands	25	2.5568[a]	0.53970	0.10794
	SRV	15	2.5600[a]	0.24113	0.06226
	Total	**90**	**2.4752**	**0.37503**	**0.03953**

EZ = ecological zone, N = number experimental units, Std. Dev = standard deviation, Std. Error = Standard error. Means with similar superscripts ([a,b]) are not significantly different ($p \geq 0.05$).

4.4. Discussion

The McMaster FEC method is the first line of GIP screening and quantification in ruminant animal hosts and is a valuable screening protocol for determining the occurrence and relative intensity of infection. The FEC in Merino sheep showed a statistically significant ($p < 0.05$) difference across ecological zones, with the highest infection rates (1170.97 ± 113.134) recorded in the Highlands. The lowlands recorded the lowest rates of strongyles (183.63 ± 39.911). Farms in the Highlands seemingly had higher stocking rates than those in the lowlands. Moreover, the Lowlands are nearer to veterinary facilities for timely treatments and animals are kept in the villages while the Highlands take their animals into hard-to-reach mountain animal posts during summer away from essential services. These conditions incline to exacerbate the intensity of parasites in the Highlands. Our survey detected burdens and prevalences of parasites comparable to findings by Moiloa *et al.* (2019), Matsepe *et al.* (2021), Mahlehla *et al.* (2021), and Phalatsi *et al.* (2022) in Lesotho. Mpofu *et al.* (2020) recorded high prevalence and parasite burdens in sheep in the summer and attributed them to the rainy season. The present results revealed strongyles were the most prevalent and abundant GIP in Merino sheep across Lesotho. The GIP prevalence dynamics of the present

study were similar to those of Moiloa *et al.* (2020); Matsepe *et al.* (2021) and Mahlehla *et al.* (2021), who reported GINs as the most prevalent GIPs in small ruminants. Alternatively, Phalatsi *et al.* (2022) reported that *Eimeria*l parasites had the highest burdens and prevalence, though strongyles were still significantly high in the Maseru district, Lesotho. Notwithstanding, the prevalence rates of strongyles were still comparable (61.7%), with an overall prevalence of 64% across the four ecological zones in the present study. In another similar survey on sheep and goats, Zeryehun *et al.* (2012) recorded 67.75 and 55.47% of strongyles, respectively, in south-eastern Ethiopia. The strongyles prevalence exhibited a statistically significant difference ($p < 0.05$) across ecological zones, with the Highlands scoring the highest (89 ± 0.029%). Alternatively, the Lowlands had the lowest prevalence (41 ± 0.038%). The survey was carried out in December and January, a summer period in Lesotho presumed favourable for high proliferation and parasite activity and therefore evidenced by relatively high infection rates owing to high temperatures and humidity (Phalatsi *et al.*, 2022). Moreover, summers are also characterized by relatively good vegetation cover, which provides an ideal microenvironment for off-host larval survival and optimal sustenance on rangelands (Rajarajan *et al.*, 2017; Mpofu *et al.*, 2020). A direct correlation between strongyle burdens and prevalence in the present study is similar to that of Veena *et al.* (2020), demonstrating low intensity of GINs where there were low prevalence rates in sheep. The strongyles' direct lifecycle has also been associated with the fast escalation of infection, especially during warm months and rainy seasons when life cycles are shortened (Molento *et al.* 2016).

Eimeria spp. in the present study was the second most prevalent parasite, with an overall prevalence rate of 18%, while the Highlands and Foothills both recorded 27%. The Lowlands had the lowest (6%). There was a significant ($p < 0.05$) difference in *Eimeria*l spp. prevalence across ecological zones, but not in parasite intensity ($p > 0.05$). Contrary to the findings of the present study, Phalatsi *et al.* (2022) and Matsepe *et al.* (2021) longitudinal studies recorded lambs with significantly higher ($p < 0.05$) *Eimeria* spp. infection than adult hosts. Point prevalence as reported in the present undertaking is different when compared with longitudinal surveys. Velusamy *et al.* (2015) and Phalatsi *et al.* (2022) GIP surveys in India and Lesotho portrayed *Eimeria* spp. as the most prevalent in goats and sheep respectively and different from the findings of the present study in which, GINs were

predominant. Consequently, there was no significant difference ($p > 0.05$) in the intensity and prevalence of *Eimeria* spp. among age groups in the present study.

Monezia spp. was the least prevalent GIP, recording the least burdens and prevalence, and did not show any statistical difference ($p > 0.05$) by age or across ecological zones. *Monezia* spp. had consistently been third in prevalence and infection intensity in previous studies. The lower relative prevalence and burdens of *Monezia* spp. compared to *Eimeria* spp. and strongyles may be due to the differences in their life cycles. *Monezia*'s lifecycle requires an arthropod intermediate host which probably lengthens its prepatent period and generation interval, resulting in lower infection rates. Though not significant ($p > 0.05$), *Monezia* spp. showed more prevalence in lambs than in older sheep. Lambs get infected by the eggs from contaminated milk and ewes' udders while they suckle. This corroborated Fagbemi and Dipeolu's (1983) records, where a similar trend prevailed in other parts of Nigeria, in which juvenile animals had more tapeworm burdens than their adult counterparts did.

Morphometrical characterization of L_3 is a common protocol used for speedy GIP diagnosis and is also valuable for determining epidemiological-based factors such as parasite intensity and transmissibility of GIN within the host populations. Over the years, little has changed in the morphological identification principle of the GINs of ruminants. van Wyk and Mayhew (2013) compiled a practical approach to the identity of GINs' L_3 using comparative morphological and morphometric guides. The protocol uses structures such as cranial extremities (head shape), the number of intestinal cells, and the sheath tail extension (STE) length value (´X´) as the main distinctive traits for differentiation. The protocols' development over the years has also provided a distinction between the specific identity of GINs and their specific hosts in an easy-to-use manual and went further to epitomise parasite-host evolutionary relationships.

The ultimate differential diagnosis between infective larvae of *H. contortus* and *H. placei*, as close relatives, became a morphological identification and diagnostic breakthrough, as they were previously difficult to differentiate. The present study recorded the length of STE as a mean of 81.7±1.3 µm and 2.4752±0.03953. All the L_3s identified had a characteristic bullet-shaped head. van Wyk *et al.* (2004) recorded an average STE length of 74 µm and estimated the ´X´ value for *H. contortus* to range between 2.2 and 2.7. ´X´ is a comparative value calculated from an equation using the STE in *Trichostrongylus columbriformis* for

relativity. Moudgil *et al.* (2022) measurements of *H. contortus* yielded STE of 71.9± 2.8 µm. Though the STE average length of *H. contortus* in the present study was relatively longer, the calculated ´X´ value fell within the range prescribed by van Wyk and Mayhew (2013). The length of STE did not show a statistical difference ($p > 0.05$) between ecological zone populations in the present study. Matsepe *et al.* (2021) revealed *H. contortus* was the sole strongyle infecting goats by using morphological features as per the van Wyk and Mayhew (2013) protocol. Abu-Elwafa *et al.* (2016) study on the identification of nematodes of ruminants revealed *H. contortus* L_3 from sheep as medium-sized, measuring 680–800µm, round-headed, and with a kinked STE of 68–80 µm. Differential diagnosis ruled out *H. placei* due to its longer mean STE (102 µm) and higher range (80-119 µm). On the other hand, Cooperia has a somewhat similar STE range to *H. contortus*, except for the difference in the overall length of the L_3 and the shape of the 'head' region. Similar morphological features, such as a bullet-shaped anterior end and kinked tail, were recorded by Veena *et al.* (2020) for a couple of GINs, including *H. contortus*. The result gives confidence in the identification of *H. contortus* in the present study. The difference in the length of L_3 could have arisen from the difference in the length of incubation to harvest of larvae and the general experimental conditions employed by different scholars.

4.5. Conclusion

The three GIPs diagnosed in Merino sheep in the four ecological zones of Lesotho comprised strongyles, *Monezia* spp., and *Eimeria* spp. The strongyles were the most prevalent and ubiquitous GIP group across Lesotho. The topographical distribution of GIP showed significant ($p<0.05$) differences between strongyles and *Eimeria* spp with the Highlands experiencing higher parasite intensities. Consequently, detecting different GIPs and their different intensities. Therefore, the null hypothesis (Ho) that there is no significant variation of GIPs in Merino sheep in four ecological zones in Lesotho is consequently rejected. The current study also revealed no significant difference in parasitic prevalence or intensity between the three sheep age groups. *Haemonchus contortus* was identified as the predominant gastrointestinal nematode in Merino sheep, with 100% occurrence rate, confirmed through the morphometric characterisation of the third-stage. The result portrays *H. contortus* infection dominance as the major cause of helminthiasis in Merino sheep in Lesotho.

CHAPTER 5

Molecular characterization of *Haemonchus contortus* (Nematoda: Trichostrongylidae) based on the Second Internal Transcribed Spacer gene of the Ribosomal DNA from Merino sheep in Lesotho

5.1. Introduction

Parasitic infections and substandard husbandry practices are major threats to a successful small-ruminant industry across the globe (Dey *et al.*, 2019). Gastrointestinal parasites (GIP) are some of the most common infections in ruminants that impact livestock production negatively. Moreover, the costs of GIN control and subsequent management of anthelmintic resistance (AR) upsurge the overall economic losses to the industry (Iqbal *et al.*, 2005; Munguía-Xóchihua *et al.*, 2018). The most disreputable GIN that infects small ruminants is *H. contortus* (the barber's pole worm). *Haemonchus contortus* is mainly prevalent in tropical and subtropical areas, especially the resource-poor communities of Africa, including Lesotho (Lichtenfels *et al.*, 1994; Troell *et al.*, 2006; Murad *et al.*, 2018; Dey *et al.*, 2019; Mahlehla *et al.*, 2021).

The importance of correct disease diagnosis cannot be overemphasised because it provides valuable information on the identification of disease agents (Molento *et al.*, 2016). Moreover, accurate disease diagnosis may lead to strategic and appropriate parasite management and control response. For the past several decades, microscopy has been successfully used in the laboratory diagnosis of pathogenic nematodes, screening eggs from faecal material, and providing a general parasitic viewpoint with limited species identity (van Wyk and Mayhew, 2013). Comparative morphological identification of adult and larval nematodes to genus and species level was also useful in laboratory helminth diagnostics though laborious (Heise *et al.*, 1999; van Wyk and Mayhew, 2013; Zarlenga *et al.*, 2016; Matsepe *et al.*, 2021). The morbidity and mortality caused by closely related GINs require extensive knowledge for appropriate differential diagnosis, hence the need for search into

more sensitive methods of pathogen identification. Morphological identification between *H. placei* parasitizing cattle and *H. contortus* in sheep posed a challenge of precise distinctions, especially where the two hosts co-exist in grazing rangelands (Zarlenga *et al.*, 2016).

A molecular protocol for parasite identification was introduced as an additional method of disease diagnosis that proved effective and more accurate (Emery *et al.*, 2016). Molecular techniques utilise genetic markers to accurately identify all life stages from genus to sub-species level in one polymerase chain reaction (Knoll *et al.*, 2021). Molecular and morphological diagnosis were used simultaneously to identify parasites effectively, timeously, and accurately from various epidemiological perspectives (Mangkit *et al.*, 2014). Several studies conducted using microscopy revealed the presence of nematodes, coccidia, and tapeworms as the three main GIPs infecting small ruminants in Lesotho (Mahleha *et al.*, 2021). The present cross-sectional study was conducted to determine the prevalence of GIPs based on faecal egg morphology and the characterization of the GIN L_3. Furthermore, the molecular population genetics of *H. contortus* from four ecological zones of Lesotho were investigated using the ITS-2 segment of the ribosomal gene.

5.2. Materials and Methods

5.2.1. Experimental Design

Coprocultures were pooled and arranged per collection site. L_3 suspensions were then morphologically identified and divided into groups of 5–10 larvae in 1.5-ml Eppendorf tubes. To break the cell structure, the L_3 suspensions were frozen at -80 and thawed at 50 °C twice consecutively for 20 minutes at each temperature. The L_3 suspension was then centrifuged at 14,000 rpm for 10 minutes to produce a pallet. The supernatant was discarded without tempering the pallet. The pallet was homogenised using an Eppendorf homogeniser, resuspended in PBS, and stored temporarily at -20 °C for DNA isolation. DNA was extracted, and amplicons were checked for purity, quality, and concentration by 1.5% gel electrophoresis and nanodrop simultaneously. Amplicons were then purified by column purification and prepared and sent to Germany for sequencing.

5.2.2. Larval DNA extraction

DNA was extracted from the L_3 homogenates using the QIAamp1 DNA mini kit as per the manufacturer's instructions. 200 µL of larval homogenate was mixed with 800 µL of RTL lysis buffer and left at 4°C overnight. 200 µL of Buffer AL was added to 200 µL of homogenized lysate in RLT Plus buffer. 400 µL of 90% ethanol was added to 400 µL (v/v) and mixed thoroughly by pipetting. The procedure was repeated until all lysate was used. 800 µL of the sample product was transferred into a DNeasy mini spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at 8000 rpm. The flow-through was discarded. 500 µL of Buffer AW1 as membrane binding buffer was added to the DNeasy spin column, centrifuged for 30 seconds at 8000 rpm, and the flow-through was discarded. The DNeasy spin column was washed with 500 µL of Buffer AW2 and centrifuged for 30 seconds. The flow-through was discarded, the collection tube changed, and the DNeasy spin column was centrifuged at full speed for 1 minute to dry the spin column. 1.5 ml of Eppendorf tube was then introduced, and 50 µL of DNA-free water was added in the middle of the spin column membrane, which was closed, and incubated for at least 5 minutes at 15–25 °C. The spin column was then centrifuged for 1 minute at 8000 rpm to elute DNA. Eluted DNA was then stored at -20°C for further downstream PCR analysis.

5.2.3. PCR amplification

To confirm the studied GIN positively as *H. contortus*, PCR amplification of the conserved ITS-2 segment of the ribosomal DNA of *H. contortus* was done using oligonucleotide primers NC1F: 5'-ACGTCTGGTTCAGGGTTGT-3' and NC2R: 5'-TTAGTTTCTTTTCCTCCGCT-3'. (Stevenson, 1995; Dey *et al.*, 2019). A PCR mix of a total volume of 25 µL that comprised 10 µL of DNA-free water, 0.25 µL of each primer (10 µM), 12.5 supermix, and 2.0 µl of DNA template (30 – 100 ng/µL) was performed. PCR conditions were carried out in an automated Biorad thermocycler as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 60 seconds, with a final elongation step at 72 °C for 7 min. To evaluate the amplification, PCR amplicons were run on 1.5% gel electrophoresis and measured against a 100 bp ladder. All bands measured about 350 bp, which was characteristic of *H. contortus*.

5.2.4. DNA purification and sequencing

Positive gel bands and amplicons were column purified using the QIAquick PCR purification kit following manufacturer instructions. The purified amplicons quality and concentration were analysed on 1.5% gel electrophoresis and a nanodrop. The purified amplicons were then labelled, packaged, and sent for Sanger sequencing services in Germany. Amplicons were sent in pairs, each with a forward and reverse primer aliquot of 14 µL of template/primer mix (4 µL = primer and 10 µL = template). The resulting sequences were deposited in GenBank and were assigned accession numbers. The consensus sequences were assembled from two complementing forward and reverse fragments. DNA Consensus sequences were then assembled and edited from forward and reverse sequences using Staden package software, version 2.0.0 (Staden *et al.*, 2000).

5.2.5. Data analysis

All sequences in the study were aligned and analysed for identification in the nucleotide blast programme of the National Centre for Biotechnology Information (NCBI) (http://www.NCBI.nlm.gov/BLAST). The best substitution model for phylogenetic tree analysis was determined using Jmodel Test version 2.1.10 software (Darriba *et al.*, 2012; Guindon and Gascuel, 2003). The method entailed the selection of the model with the lowest Bayesian information criterion (BIC). The phylogenetic tree with bootstrap tests and pairwise alignment of estimates of evolutionary divergence were assembled in MEGA11 software (Tamura *et al.*, 2021). DNAsp version 6.12.03 and Arlequin version 3.5 (Rozas *et al.*, 2017; Excoffier *et al.*, 2010) were used to determine molecular indices comprising nucleotide diversity (π), haplotype diversity (Hd), sequence polymorphism analysis, and neutrality test values. ITS-2 gene is a molecular marker used mainly for molecular identification assessing genetic diversity, and mapping gene flow among GIN populations. Haplotype diversity was calculated as a measure of variation and distribution of specific combinations of alleles within a population. DNAsp version 6.12.03 package was used to evaluate Haplotype diversity, an index used to estimate the probability of at least two randomly chosen haplotypes being different.

Haplotype diversity (Hd)

- $Hd = n (1 – \Sigma Pi^2) / (n – 1)$
- Where;
- n = number of populations,
- Pi = haplotype frequency in the tested population

Nucleotide diversity estimates the genetic diversity at the nucleotide level of a particular population based on pairwise comparison of the sequences.

Nucleotide diversity, Pi(π)

- $\pi x = n / (n – 1) \Sigma xi\ xj\ \pi ij$
- Where:
- n = number of sequences under evaluation
- xi = estimated frequency of the ith sequence in a population
- xj = estimated frequency of the jth sequence in a population
- π = proportion of different nucleotides between sequences i and j.

DNAsp version 6.12.03 was also used to compute the allelic variations between the parasite populations in the study area. The fixation index (F_{ST}) was calculated in the present study as a measure of population differentiation.

- F_{ST} formula
- $F_{ST} = (HT – HS)/HT$
- Where:
- HS = average heterozygosity in a sub-population
- HT = average heterozygosity in a metapopulation
- F_{ST} = the reduction in heterozygosity that is due to the structure of the population

Genetic diversity within and among populations of the four ecological zones of Lesotho was determined by an analysis of molecular variance (AMOVA) using the Arlequin version 3.5 software package (Excoffier *et al.*, 2010). Haplotype network analysis based on a median-joining algorithm signifying the genetic distances between Lesotho isolates and 18 other sequences from NCBI was constructed using Popart version 1.7 (Bandelt *et al.*, 1999).

5.3. Results

5.3.1. Molecular characterization of H. contortus L_3 based on ITS-2

The *H. contortus* ITS-2 gene segment of the ribosomal DNA was analyzed for identity, genetic diversity, and determination of population structure in the selected four ecological zones of Lesotho. A total of 35 L_3 initially identified morphologically as *H. contortus* were prepared for PCR amplification. Figure 5.1. depicts 350 bp single bands of *H. contortus* amplicons on 1.5% agarose gel electrophoresis. Isolate consensus sequences were verified on the NCBI database and positively identified as comprising a partial 5.8S gene, a complete ITS-2 gene with a portion of the large subunit gene of *H. contortus.*

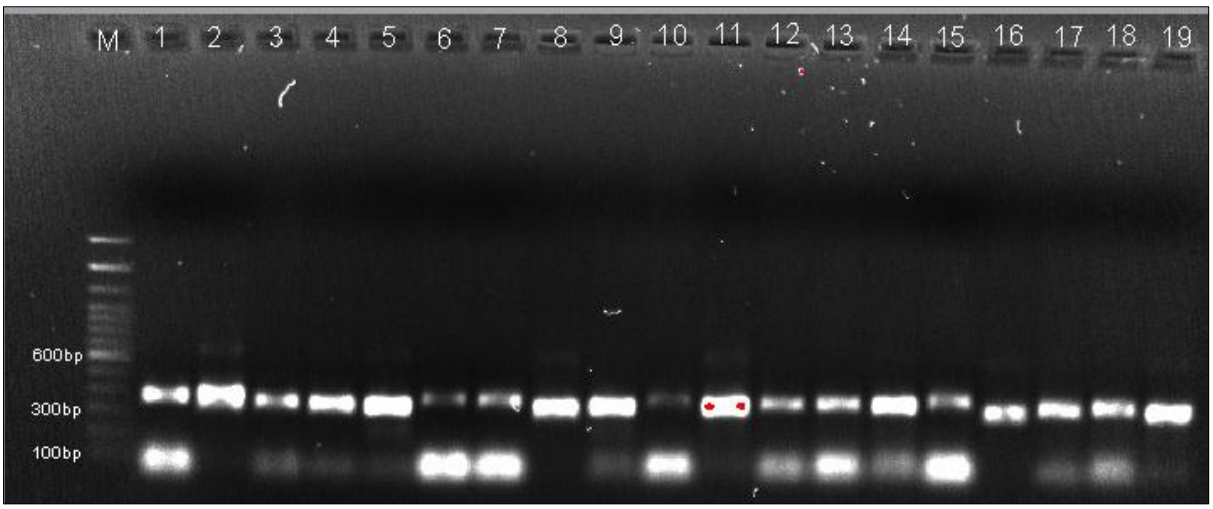

Figure 5.1. Agarose gel electrophoresis of *H. contortus* larvae amplicons from the study. The gel image shows a PCR product of ±350 bp at 1.5% agarose, 100 volts for 50 minutes. A ladder of 100 bp was used. Samples 1 -5 were from Highlands, 6- 10 from Foothills, 11-17 from the Lowlands, and 18 and 19 were from the Senqu River valley.

The nucleotide homology of all sequences in the study ranged between 97 and 99%. The overall G+C content of the sequences in the study was 32.9%. The nucleotide analysis revealed 224 monomorphic sites and six polymorphic sites. There were five singleton variables on positions: 66, 68, 79, 203, and 236, while the Parsimony informative sites were three on positions 18, 21, and 22. Table 5.1 displays nucleotide information on the polymorphic sites of the ITS-2 of *H. contortus* isolates using X78803 as a reference. There were two types of transversion substitution mutations of T <->A on positions 17, 79, and

201, while C <-> G was on position 21. Transition mutations of C <->T change were on position 126. To rule out the *H. placei* as a possible identity of Lesotho isolates, they were further aligned with the reference sequence, X78812. The alignment revealed three purine transitions at nucleotide positions 24 (G), 205 (A), and 219 (G), which were characterized as species-specific SNP for *H. placei* as defined by Stevenson *et al.* (1995) and Chaudhry *et al.* (2014). In addition to the species-specific SNPs above, alignment revealed a unique transition mutation (C/T) in all Lesotho isolates at nucleotide position 123rd with X78812 and X78803.

Table 5.1: Nucleotide information depicting polymorphic sites of the ITS-2 of *H. contortus* isolates from 20 sequences from four ecological zones of Lesotho using X78803 as a reference segment. A dash portrays an identical nucleotide to the reference gene sequence.

Collection site	Accession Number	District	EZ	Nucleotide Position							
				17	20	66	68	79	99	126	201
Reference sequence	X78803			T	C	T	C	T	C	C	T
Mokotane	OQ674238			-	-	-	-	-	-	T	
Ha Letuka	OQ674239			-	-	-	-	-	-	T	T
Ha Thabo	OQ674240			-	-	-	-	-	-	T	T
Macheseng	OR523097			-	G	-	-	-	-	T	T
Thabamoea	OR523098			-	-	-	-	-	-	T	T
Pitseng	OQ674241	LR	FH	-	G	-	-	-	-	T	T
Tsunyane	OQ674242			A	G	-	-	-	G	T	-
Matlameng	OQ674243			-	G	C	A	-	-	T	T
Ha makape	OQ674244			-	-	-	-	-	-	T	T
Mphosong	OQ674245			-	G	C	A	-	-	T	T
Ha Mohlalefi	OQ674246	MF	LL	-	-	-	-	-	-	T	T
Makintane	OR523099			-	G	-	-	-	-	T	T
Hermoni	OR523100			-	-	-	-	-	-	T	T
Ha Mokhalinyane	OQ674247	MS		-	G	-	-	A	-	T	T
Moeletsi	OQ674248	MF		-	G	-	-	-	-	T	-
Mphaki	OQ674249	QT	SRV	-	G	C	A	-	-	T	T
Qhoalinyane	OQ674250			A	G	-	-	-	-	T	-
Ha Sekake	OQ674251	QN		-	-	-	-	-	-	T	T
Patlong	OR523101			-	G	-	-	-	-	T	T
Makanyane	OR523102			-	G	-	-	-	-	T	T

EZ=Ecological Zone, TT=Thaba Tseka, LR=Leribe, MF=Mafeteng, MS=Maseru, QT=Quthing, QN=Qacha's Nek, HL=Highlands, FH=Foothills, LL=Lowlands, SRV=Senqu River Valley. T = Thyamine, G = Guanine, C = Cytosine, A = Adennine

5.3.2. Phylogenetic analysis and Genetic diversity of Haemonchus contortus based ITS-2 gene of the ribosomal DNA in Lesotho

For the determination of the phylogenetic status of *H. contortus*, 20 isolates from the present study and 18 other world sequences from four continents (Africa, Asia, Europe, and Oceania) were selected from the NCBI and compared in the analysis (Table 5.2). Figure 5.2 presents a phylogenetic analysis of Lesotho isolates with global sequences based on 231 bp of the ITS-2 complete gene of *H. contortus* (Tamura *et al.*, 2021). The highest bootstrap value in the tree was 64%, with 54% the highest among Lesotho isolates. All nodes that exhibited less than 50% bootstrap values were not included in the final phylogenetic tree inference. The partial gene sequence of *Trichostrongylus columbriformis* ITS-2 (accession number: AB908960) was used as an out-group. The phylogenetic tree revealed two major clades (A and B) and three subclades (C, D, and E). All Lesotho isolates clustered in Clade A which included sequences from Lesotho, Asia, and Africa, and one from Oceania. Clade C consisted of eight Lesotho isolates. The distribution of Lesotho isolates among the clades did not show geographic or topographic inclination. Table 5. presents global *H. contortus* ITS-2 sequences from NCBI used in the inferred phylogenetic tree with Lesotho isolates.

A pairwise alignment representing estimates of evolutionary divergence between 20 isolates of *H. contortus* sequences from different ecological zones of Lesotho is shown in Table 5.3. The pairwise evolutionary divergence revealed very low variabilities in the complete ITS-2 gene, ranging from 0.0000 to 0.02228. The pairwise nucleotide diversity was low across the Lesotho *H. contortus* populations. The highest pairwise value (0.2228) was between the SRV isolate and six other isolates from the Highlands, Lowlands, and Foothills (OQ674238, OQ674239, OQ674240, OQ674246, OQ674244, and OQ674251).

Table 5.2: Global ITS-2 gene sequences based on 231 bp from the GenBank used for phylogenetic analysis and Haplotype network analysis with Lesotho isolates in the present study. Sequences are presented with their accession numbers, host species, life cycle stage, country of origin, and haplotype number in the haplotype network analysis

	Genbank Accession Number	Host Species	Life Cycle Stage	Country Of Origin
1	X78803	-	Adult/adult/egg	Australia
2	OM327424	-	-	India
3	OQ401457	-	Adult	Iran
4	KP688065	African buffalo	Adult	South Africa
5	MT193663	Goat	Larval	China
6	AB908961	Goat	Adult	Japan
7	KC998713	Sheep	Larvae	New Zealand
8	KC998714	Sheep	Larval	New Zealand
9	AB682686	Sheep	Adult	Egypt
10	MT568605	Sheep	Larval	Myanmar
11	LC368060	Goat	Adult	Nigeria
12	MN708983	Sheep	Adult	Cameroon
13	MH481595	Cattle	Larval	Ghana
14	AB682687	Sheep	Adult	Egypt
15	KP760874	Blue Wildebeest	-	Kenya
16	JF680983	Sheep	Adult	Ireland
17	AB908963	Goat	Adult	Laos
18	KM586652	-	Larval	China
19	LC360154	Goat	-	Bangladesh
20	KX829170	Cow	-	Iran
21	OR702552 *H. Placei*	-	-	Nigeria
22	AB908960, *T. Columbriformis*	Goat	Adult	Laos

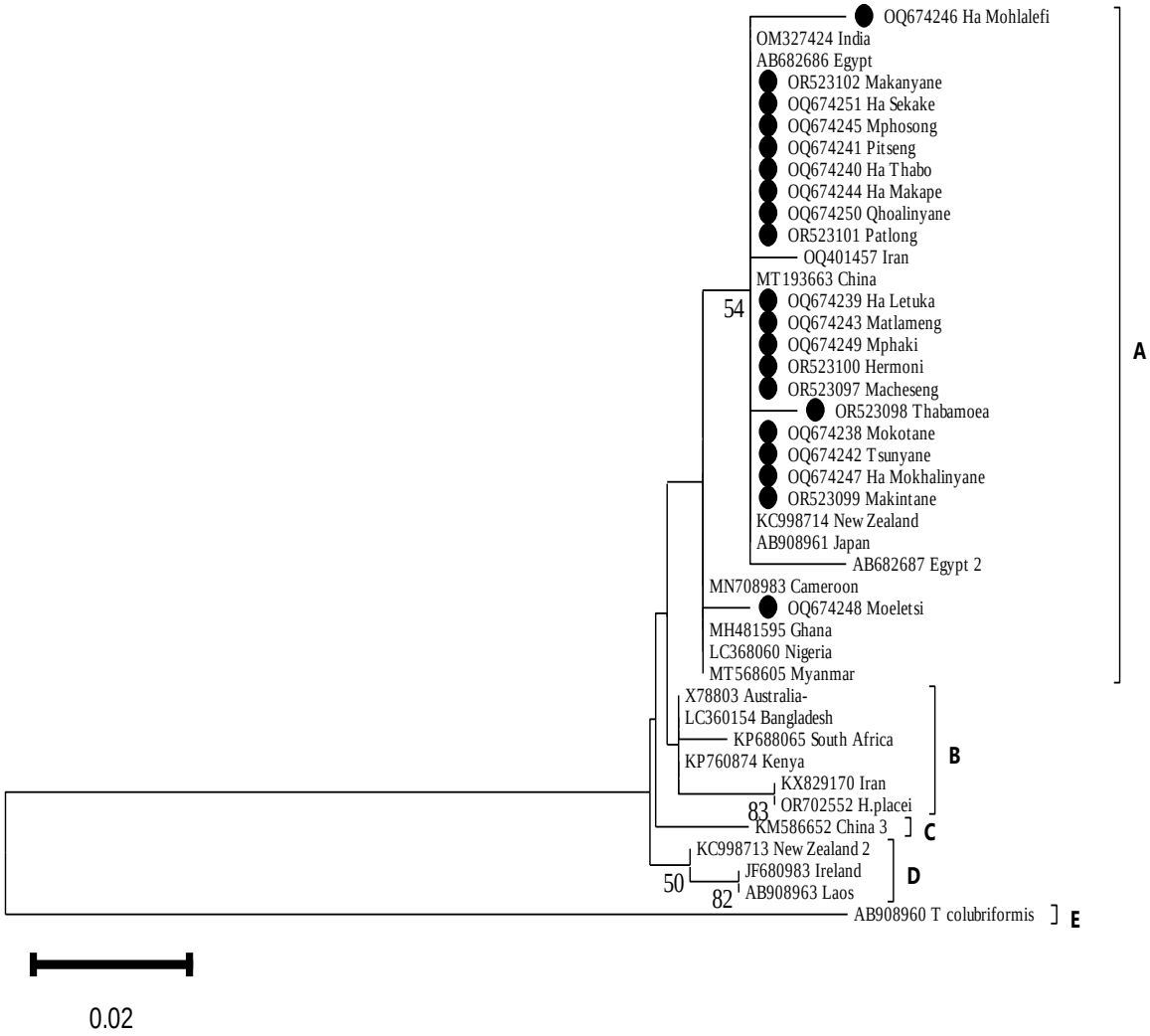

Figure 5.2: Phylogenetic tree analysis showing the evolutionary history constructed using the Neighbor-Joining method (Saitou, and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992) and are in the units of the number of base substitutions per site. This analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). Lesotho isolates are marked with black dots. There was a total of 165 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura, Stecher, and Kumar, 2021)

5.3.3. Population genetic structure of Haemonchus contortus based on ITS-2 in Lesotho

Haplotype diversity was used in population genetics to study the genetic variation within a population. The Lesotho isolates based on ITS-2 of the ribosomal DNA analysed in DNAsp v6 and Arlequin 3.5.18 revealed four distinctive haplotypes (Excoffer and Lischer, 2010). The molecular indices depicting the intra-population dynamics of the Lesotho isolates for four ecological zones are presented in Table 5.4. The average haplotype diversity and nucleotide diversity values in our current study were 0.7684 and 0.00590, respectively. The Lowlands and SRV had haplotype diversity of 0.9000 and 0.8000. Highlands and foothills both had the lowest haplotype diversity of 0.7000 in the study.

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Table 5.3: Estimates of Evolutionary Divergence between 20 isolates sequences from four ecological zones of Lesotho

ITS-2 Isolates	OQ674238	OQ674239	OQ674240	OQ674241	OQ674242	OQ674243	OQ674244	OQ674245	OQ674246	OQ674247	OQ674248	OQ674249	OQ674250	OQ674251	OR523097	OR523098	OR523099	OR523100	OR523101	OR523102
Mokotane_OQ674238																				
Haletuka_OQ674239	**0.0000**																			
Hathabo_OQ674240	**0.0000**	**0.0000**																		
Pitseng_OQ674241	**0.0044**	**0.0044**	**0.0044**																	
Tsunyane_OQ674242	**0.0088**	**0.0088**	**0.0088**	**0.0044**																
Matlameng_OQ674243	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**															
Hamakape_OQ674244	**0.0000**	**0.0000**	**0.0000**	**0.0044**	**0.0088**	**0.0044**														
Mphosong_OQ74245	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0040**	**0.0000**	**0.0044**													
Hamohlalefi_OQ674246	**0.0088**	**0.0088**	**0.0088**	**0.0133**	**0.0178**	**0.0133**	**0.0008**	**0.0133**												
Hamokhalinyane_OQ674247	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0133**											
Moeletsi_OQ674248	**0.0133**	**0.0133**	**0.0133**	**0.0088**	**0.0133**	**0.0088**	**0.0133**	**0.0088**	**0.0223**	**0.0088**										
Mphaki_OQ674249	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0133**	**0.0000**	**0.0088**									
Qhoalinyane_OQ674250	**0.0088**	**0.0088**	**0.0088**	**0.0044**	**0.0000**	**0.0044**	**0.0088**	**0.0044**	**0.0178**	**0.0044**	**0.0133**	**0.0044**								
Hasekake_OQ674251	**0.0000**	**0.0000**	**0.0000**	**0.0044**	**0.0088**	**0.0044**	**0.0000**	**0.0044**	**0.0088**	**0.0044**	**0.0133**	**0.0044**	**0.0088**							
Macheseng_OQ523097	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0133**	**0.0000**	**0.0088**	**0.0000**	**0.0044**	**0.0044**						
Thabamoea_OQ523098	**0.0088**	**0.0088**	**0.0088**	**0.0133**	**0.0178**	**0.0133**	**0.0088**	**0.0133**	**0.0178**	**0.0133**	**0.0223**	**0.0133**	**0.0178**	**0.0088**	**0.0133**					
Makintane_OQ523099	**0.0088**	**0.0088**	**0.0088**	**0.0044**	**0.0088**	**0.0044**	**0.0088**	**0.0044**	**0.0178**	**0.0044**	**0.0133**	**0.0044**	**0.0088**	**0.0088**	**0.0044**	**0.0088**				
Hermoni_OQ523100	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0133**	**0.0000**	**0.0088**	**0.0000**	**0.0044**	**0.0044**	**0.0000**	**0.0133**	**0.0044**			
Patlong_OQ523101	**0.0000**	**0.0000**	**0.0000**	**0.0044**	**0.0088**	**0.0044**	**0.0000**	**0.0044**	**0.0088**	**0.0044**	**0.0133**	**0.0044**	**0.0088**	**0.0000**	**0.0044**	**0.0088**	**0.0088**	**0.0044**		
Makanyane_OQ523102	**0.0044**	**0.0044**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0044**	**0.0000**	**0.0133**	**0.0000**	**0.0088**	**0.0000**	**0.0044**	**0.0044**	**0.0000**	**0.0133**	**0.0044**	**0.0000**	**0.0044**	

The Number of Base Substitutions Per Site From Between Sequences is shown. Analyses Were Conducted Using The Tamura 3-Parameter Model. This Analysis Involved 20 Nucleotide Sequences. All Positions Containing Gaps and Missing Data were liminated (Complete Deletion Option). There were a total of 228 Positions in The Final Dataset. Evolutionary Analyses were Conducted In Mega11

Table 5.4: Molecular indices of *H. contortus* populations based on the partial segment of the Second Internal Transcribed Spacer of the ribosomal gene from four ecological zones of Lesotho

EZ	N	NPS	H	SS	Hd	Pi	MNPD	SSF	T's D	Fu's Fs
Highlands	5	3	3	3	0.7000 ± 0.2184	0.00519	1.2 ± 0.9085	0.44	-1.04849	-0.18585
Foothills	5	3	3	2	0.7000 ± 0.2184	0.00351	1.2 ± 0.9085	0.44	-0.97256	-0.18585
Lowlands	5	11	4	4	0.900 0± 0.1610	0.00702	4.6 ± 2.7129	0.28	-1.14554	0.35685
SRV	5	3	3	2	0.8000 ± 0.1640	0.00439	1.4 ± 1.0189	0.36	0.24314	0.06069
MTT	20	14	4	6	0.7684 ± 0.0689	0.00519	2.16 ± 1.252	0.27	-1.30145	-0.96565

Nb: EZ = Ecological Zone, SRV = Senqu River Valley, N = Number of Sequences, NPS = Number of Polymorphic Sites, H = Number of Haplotypes, SS = Sites with substitutions, Hd = Haplotype Diversity, PI = Nucleotide Diversity, MNPD = Mean No. Pairwise Differences, SSF= Sum of Square Frequencies, MTT = Mean Total. T'sD = Tajima's D Value, Fu's Fs = Fu's Fs Neutrality Test

Genetic differentiation of *H. contortus* L_3 isolates among ecological zone populations was established through pairwise F_{ST} values (Table 5.5). The highest inter-population genetic differentiation (F_{ST} = 0.21875) was recorded between pairwise comparison between Highlands and Foothills while the least genetic dissimilarity (F_{ST} = -0.18427) was recorded between the Lowlands and the SRV. The negative pairwise F_{ST} values are regarded same as zero. The F_{ST} values between the Foothills, Lowlands, and SRV are all negative. Low levels of genetic differentiation among *H. contortus* isolates are indicative of relatively high levels of gene flow among the populations. The values for Tajima's D and Fu's Fs tests were -1.30145 and -0.96565 respectively. The neutrality tests revealed no significant deviation ($p > 0.05$) from neutrality within the parasite sub-populations. Analysis of molecular variance (AMOVA) was calculated to determine gene flow within and among ecological zone populations. The majority of molecular variation had distribution within topographic populations at 94.35 % while 5.65% was among ecological zone populations (F_{ST}=0.05651, $p > 0.05$).

Table 5.5: Population Pairwise F_{ST} values of *H. contortus* L_3 from four ecological zones of Lesotho based on ITS-2 of ribosomal DNA

Ecological Zone	Highlands	Foothills	Lowlands	Senqu River Valley
Highlands	0.0000	-	-	-
Foothills	0.21875	0.00000	-	-
Lowlands	0.12500	-0.07143	0.00000	-
Senqu River Valley	0.05172	-0.18427	-0.04839	0.00000

A total of 40 ITS-2 complete gene (231 bp) sequences of *H. contortus* were used to construct the median-joining haplotype analysis of Lesotho and global sequences from Africa, Asia, Europe, and Oceania retrieved from NCBI (Figure 5.3). There was a total of 13 *H. contortus* ITS-2 haplotypes from the analysis and two other out-group sequences, namely *H. placei* (OR702552) and *Teladosargia circumcinta* (AB908960). Haplotype 2 was the biggest and was shared between 22 sequences. Lesotho isolates were shared among four haplotypes in the analysis, of which 17 belonged to haplotype 2. There were 17 sequences from Lesotho and five others (KC998714-New Zealand, MT193663-China, AB682686-Egypt, AB908961-Japan, and OM327424-India) from around the world, making haplotype 2. The presentation also provides haplotype frequencies and the number of mutations between

respective haplotypes. The overall haplotype diversity and nucleotide diversity of the ITS-2 sequences from Lesotho were 0.7684 ±0.0689 and 0.00519, respectively.

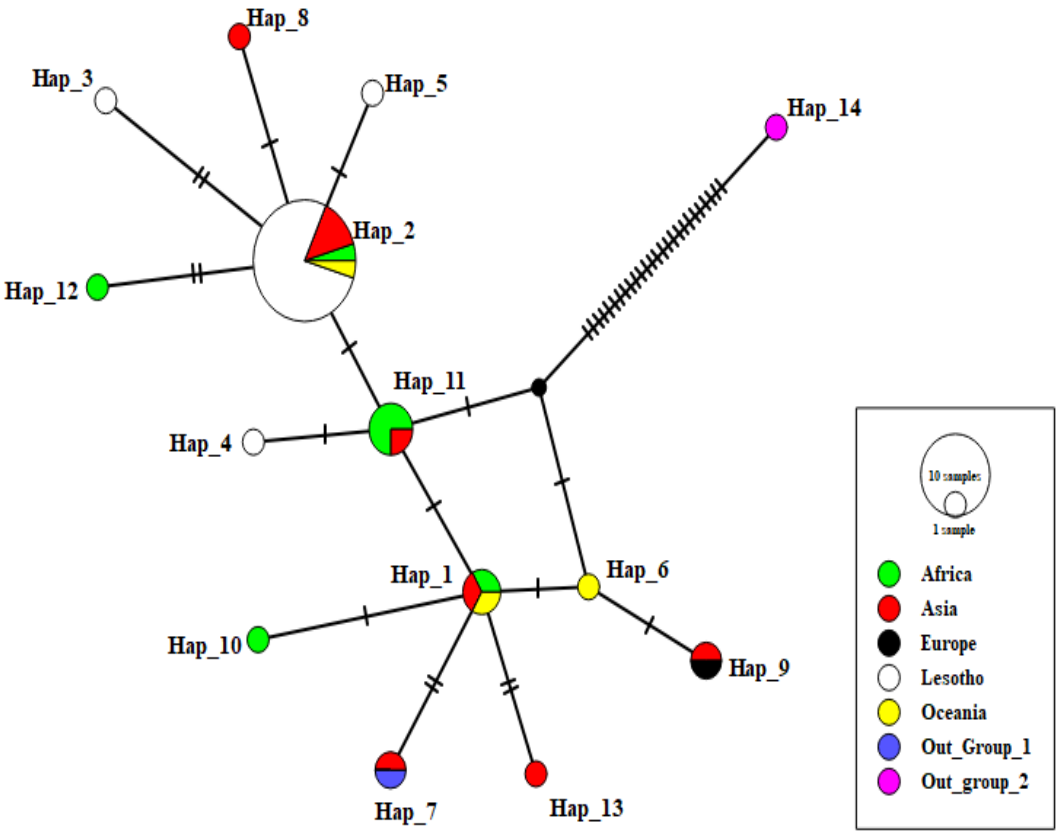

Figure 5.3: Median-joining haplotype analysis of Lesotho and selected global *H. contortus* based on ITS-2 partial gene (231 bp) sequences. 14 haplotypes resulted from overall 42 sequences. Each disc represents a haplotype; the disc size depicts the relative frequencies for each haplotype. Different colour shades represent either continental and/or Lesotho populations. Mutation changes are represented by lines between the discs. Black dots are median vectors (Bandelt *et al*. 1999).

5.4. Discussion

Molecular characterization and determination of genetic diversity in GINs have been growing in epidemiological surveys in recent years. The ribosomal DNA gene, ITS-2 has been used to identify several trichostrongylids of economic importance across the globe (Stevenson *et al.*, 1992; Mangkit *et al.*, 2014; Chaudhry *et al.*, 2015; Zhang *et al.*, 2016;

Kandil *et al.*, 2018). Consequently, the possession of conserved sites by the ITS-2 gene also makes it an ideal molecular marker for species identification, as it accurately identifies different life stages (adults, larval instars, and eggs) to species and subspecies levels (Heise *et al.*, 1999; Avramenko *et al.*, 2015; Kandil *et al.*, 2018). This molecular marker was therefore preferred due to its sensitivity, stability, and high mutation rates (Zarlenga *et al.*, 2001; Santos *et al.*, 2020; Hade *et al.*, 2022). The molecular characterization of *H. contortus* L_3, based on the ITS-2 gene of the ribosomal DNA was inaugural in Lesotho. Molecular determination has become a valuable component of epidemiological information, needed in parasite diagnostics, tracer study initiatives, phylogenetic and evolution analysis, population genetics, and the determination of gene flow within and among parasite populations (Yin *et al.*, 2016). *Haemonchus contortus* L_3s isolates prepared from coprocultures across four ecological zones of Lesotho were characterized using the morphometrical protocol by van Wyk and Mayhew (2013) and confirmed by molecular genotyping. Similarly, Alborzi *et al.* (2023) characterized *H. contortus* by morphometric and molecular methods and reported that both yielded dependable results among several GINs. The PCR amplification and subsequent gel electrophoresis were run from universal primer sets, NC1F and NC2R, and yielded ≈350 bp band images, characteristic of *H. contortus*. The band images were comparable to results from several global research (Stevenson *et al.*, 1995; Kandil *et al.*, 2017; Laosutthipong and Eardmusic, 2019) confirming the accurate identification of *H. contortus.* The close morphological similarities of eggs, L_3, and adult stages of *H. contortus* from small ruminants and cattle *H. placei* render their differentiation using microscopy challenging. Notwithstanding the relative sensitivity and accuracy of the morphological identification, molecular characterization provides a relatively faster, more reliable, more accurate, and consistent determination. Upon sequencing of Lesotho L_3 isolates and comparing them with sequences from the GenBank, a homology of 97–100% resulted. The Lesotho isolates also presented a single open reading frame compared with ITS-2 accession number X78812. A similar molecular marker analysis was used across the globe to study *H. contortus* identification and population genetics (Yin *et al.*, 2016). Hade *et al.* (2022) reported that all isolates studied in the Iraqi survey on identification were *H. contortus*, as in our study. The ITS-2 intra-specific variation in our study was comparable to the result by Mangkit *et al.* (2014), with a difference of less than 1%. Stevenson *et al.* (1995) could not find any variation among 10 *H. contortus* DNA isolates of the identical gene segment in

Australia. The assessment of Lesotho ITS-2 sequences revealed sub-populations were *H. contortus* upon pairwise analysis with other GenBank isolates. The highest percentage variation among Lesotho isolates in the pairwise comparison of evolutionary divergence in the present survey was 2.228%. The findings in the present study are comparable to those reported by Yin *et al.* (2013), in which there was an intraspecific variation of 2.6% in 152 ITS-2 *H. contortus* from different regions in China. The results, therefore, support the ITS-2 gene of the ribosomal DNA as a reliable diagnostic molecular marker and an ideal tool for differential diagnosis. The multiple alignment comparison of Lesotho isolates with reference sequences, accession numbers X78803 and X78812, revealed three purine transitions at nucleotide positions 24 (A), 205 (G), and 219 (G), with the latter reference sequence confirming the identity of *H. contortus* as reported by Stevenson *et al.* (1995) and Pitaksakulrat *et al.* (2021). The result also confirmed positive morphological identifications of the L_3 in four ecological zones of Lesotho. The extensive molecular work done on *H. contortus* and the availability of many reference genomes deposited in GenBank, make intraspecific genetic variation studies doable and ease comparisons and conclusions to be drawn for several experiments (Emery *et al.*, 2016). The analysis of the nucleotide sequences from Lesotho isolates produced a single open reading frame of ITS-2 of the nuclear ribosomal DNA among themselves and *H. contortus* ITS-2 gene sequences retrieved from GenBank. The two methods are used effectively for different levels and specific laboratory diagnostic needs.

The overall haplotype diversity (0.76923) recorded in the study is well within the range (0.609 to 0.824) recorded by Yin *et al.* (2013). There were relatively high haplotype diversity values recorded in the present study, which suggests a comparatively large number of haplotype variations within a population indicating a diverse genetic pool. Our result was relatively lower than those recorded by Dey *et al.* (2019) in Bangladesh, Qamar *et al.* (2021) in Pakistan, and Laosutthipong and Eardmusi (2019) in Thailand, that reported haplotype diversity of 0.8695, 0.846, and 0.832, respectively. Kandil *et al.* (2017) epidemiological study of genetic diversity and patterns of gene flow for Haemonchus species in small ruminants revealed that PCR and gene sequencing led to accurate taxonomic classification of different parasite genotypes. The average nucleotide diversity (0.00590) for Lesotho isolates was lower than the results from Bangladesh (0.0098), Thailand (0.017), and Pakistan (0.0214). The high haplotype diversity suggests an elevated probability of intra-genetic

diversity among Lesotho *H. contortus* subpopulations. Consequently, the Lowlands have a higher genetic variation (Hd = 0.9000) than all ecological zones, while the Highlands and Foothills have the least relatively diverse populations. The haplotype diversities in a study by Shen *et al.* (2017) on *H. contortus* from domestic animals were lower (0.719) than the present results, while those of wild blue sheep (0.787) from China were similar to our overall result. The Foothills recorded the lowest nucleotide diversity (0.00351) of the other three, while the Lowlands (0.00702) had the highest nucleotide diversity within the population. The mean nucleotide diversity recorded was 0.00519 in the present study. Stevenson *et al.* (1995), working with 10 *H. contortus* DNA isolates, did not find variations of the identical gene in Australia. The mean total nucleotide diversity in the present study was lower than those recorded by Dey *et al.* (2019) and Laosutthingpong and Eardmusic (2019), which recorded 0.0098 and 0.017, respectively. The study by Qamar *et al.* (2021) recorded much higher nucleotide diversity (0.0214) than the present study. Inversely, the study by Shen *et al.* (2017) recorded relatively lower nucleotide diversity indices of domestic sheep (0.004), which were also lower than those recorded for wild blue sheep (0.008). High intrapopulation nucleotide diversity indices implied the possibility of a large pool of genetic variants within such a population. Alternatively, low nucleotide diversity in the parasite suggests genetic homogeneity or the possibility of recent population bottlenecks. The Lesotho isolates demonstrated moderately lower homogeneity across four ecological zones when compared to other global sequences, which had relatively higher variability. Recorded nucleotide diversity values for Lesotho isolates depicted less average pairwise genetic diversity among all sample sequences. Hade *et al.* (2022) also recorded low genetic variation in *H. contortus* in Iraq using a similar molecular marker. The present situation is supported by low estimates of evolutionary divergence from pairwise alignment analysis, indicative of low intra-specific genetic variations. Lesotho sequences with zero values on the pairwise estimates for evolutionary divergence clustered together in Clade A on the phylogenetic tree. The characteristic low genetic variation in ITS-2 evident in the current study is the reason for the use of such a molecular marker for species identification. ITS-2 proved to be an ideal molecular marker for molecular diagnosis and a reliable instrument for differentiation between closely related species, even at the sub-species level (Parvin *et al*., 2024).

Factors responsible for genetic diversity and the population genetic structure of parasites include distribution by host movements, population size, environmental and geographic

conditions, life history, and gene flow (Gilleard and Redman, 2016). Ecological zones in Lesotho show distinct seasonality with marked differences in ambient temperatures and the presence of diverse vegetation (Obioha, 2010; Phalatsi *et al.*, 2022). This was revealed by haplotype and nucleotide diversities, genetic differentiation, and the different neutrality test results of sequences analysed from respective ecological zones. The system restricts animals to grazing only in demarcated micro-communal jurisdictions. Bisset *et al.* (2014) observed that grazing livestock are at constant risk for reinfection by GINs. Animal movement for economic reasons and in search of better pastures have some degree of effect on the genetic diversity and evolution of parasites. The communal rangelands are perceived as hotspots for parasitic reinfections and are consequently responsible for dynamics in gene flow within parasite populations. GIP populations are transported and spatially distributed by hosts due to the nature of their life cycle, which includes both in- and off-host stages. GINs are prone to varying environmental, biotic, and drug selection pressures that may determine the intensity and distribution of phenotypes and genotypes among parasite populations (Emery *et al.*, 2016).

Four ITS-2 haplotypes resulted from 20 sequences of the present study. Blouin *et al.* (1995), in a similar initiative, recorded 113 haplotypes from 120 sequences. Furthermore, Dey *et al.* (2019) found 77 haplotypes in 85 individual sequences, showing a common trend among *H. contortus* isolates. The present results demonstrate an apparent interrelationship between the pairwise divergence values, phylogenetic tree analysis results, and haplotype analysis. The result revealed lower divergence values and close homology, which yielded distinct monophyletic clades on the phylogenetic tree analysis. There was also high homology between Lesotho isolates and other global sequences from GenBank. The result of the present study corroborated Pitaksakulrat *et al.* (2021), on displaying insignificant geographical genetic structure among *H. contortus* isolates. The solitary characteristics of haplotypes OQ674247, OQ674224, and OR523098 suggested the possibility of novelty for these isolates. Lesotho is South Africa landlocked and portrays a closed pastoral system.

Animal grazing patterns and movement within Lesotho rangelands are guided by the land tenure system under traditional allocation and controlled by village chiefs (Mbata, 2001). Due to its vast rural and mountainous rangelands, where most small ruminants are raised, farmers from the Highlands, Foothills, and some parts of the SRV practice transhumance.

Transhumance assembles scores of animals into a no-mans-land, communal animal posts in the Highlands. The transhumance movements may, therefore, have contributed to the observed high gene flow in the study. The second haplotype (Hap_2) from the median-joining haplotype analysis included several sequences and traversed Lesotho ecological delineations. Consequently, Hap_2 also included isolates from other continents. All sequences from the Highlands isolate were identified as Hap-2. The haplotype sharing among the majority of our isolates (17 out of 20 isolates) suggested that *H. contortus* in Lesotho does not follow a definite topographical population structure. Consequently, Lesotho *H. contortus* isolates showed distinctively low genetic distances among themselves. Moreover, the phylogenetic tree analysis revealed a monophyletic clade for all Lesotho isolates including some African and global *H. contortus*. Global sequences that clustered with Lesotho isolates were from Africa (Camerron, Egypt, Ghana and Nigeria) Asia (Japan, India and Myanmar), and Oceania (New Zealand). The lack of a definite ecological population structure among Lesotho isolates renders the traditional rangeland jurisdictions of no effect owing to the high gene flow experienced in the present study. The present result was similar to Alborzi *et al.* (2023), who could not establish the clustering of *H. contortus* isolates into specific geographic delineations or host species. Low genetic variation and high gene flow characterized Lesotho *H. contortus* populations' topographic delineations, hosts' movement restrictions, and containment by different village grazing jurisdictions. Akkari *et al.* (2013) suggested a possible correlation between high genetic diversity and animal husbandry. Notwithstanding high homology among the isolates, the present study moderately supports Dey *et al.* (2019) suggestion that two distinct global geographical patterns of *H. contortus* clades were influenced more by continental isolation. Blouin *et al.* (1995) suggested that the population genetic structure of other GINs showed major diversity distributed within populations, as revealed in our current undertaking. The negative Tajima's D value suggested that the genetic diversity within a population was skewed toward the possession of a high number of rare genetic polymorphisms. This skewness was due to various factors including population expansion, balancing selection, background selection, recent selective sweeps, or demographic events. The neutrality tests revealed there was no significant deviation ($P > 0.05$) from neutrality within the parasite sub-population.

5.5. Conclusion

The morphological identification was confirmed by molecular protocols that produced clear single bands characteristic of the ITS-2 partial gene of *H. contortus*. The study also confirmed the reliability of the protocol by van Wyk and Mayhew (2013) in identifying the third larval stage of GIN in ruminants. The population genetic indices in our study were in agreement with the existence of a generally homogeneous population of *H. contortus* in Lesotho, regardless of different ecological zones and different village grazing delineations in Lesotho. Divergent evolution and mutation from other world isolates could therefore be a possibility for the marked difference as a result of geographical divide. The results of the study provided valuable insights into the patterns of parasite gene flow, which may be used in formulating parasite control strategies. The alternative hypothesis (H_1) that there was a significant genetic variation among *H. contortus* populations across ecological zones in Lesotho was demonstrated in the study. The present initiative will therefore contribute to the epidemiological data for the development of sound management and control of GINs. The information from the study will further be utilized to facilitate the investigation of phylogenetic studies and population genetics to determine host movement and gene flow.

CHAPTER 6

Genotyping beta-tubulin isotype-1 gene for the determination of SNPs associated with benzimidazole resistance in *Haemonchus contortus* from naturally infected Merino sheep in Lesotho

6.1. Introduction

Anthelmintic drugs have been the primary control strategy against gastrointestinal nematodes (GINs) in ruminants worldwide (Salle *et al.*, 2019; Kotze and Prichard, 2016; Čudeková *et al.*, 2010). Due to the success of chemical control during its initial administration, anthelmintic drug use grew intensively worldwide and consequently, incidences of anthelmintic resistance (AR) development from GINs of small ruminants were reported for all major groups of drugs (Gilleard and Beech, 2007; Sutherland and Leathwick, 2011; Zhang *et al.*, 2016; Moharanj *et al.*, 2017;). Mahlehla *et al.* (2021) reported more than 90% of smallholder Merino sheep farmers used anthelmintic drugs to control GIN in Maseru and Quthing districts, in Lesotho. Small ruminants primarily depend on communal pastures for daily nutritional requirements in Lesotho (Moiloa *et al.*, 2020; Mahlehla *et al.*, 2021). High prevalence and intensity of GIN infections were diagnosed in small ruminants, where *H. contortus* was identified as the overwhelmingly most abundant species in four ecological zones of Lesotho (Matsepe, 2021). *Haemonchus contortus* was reported as a major cause of helminthiasis and the most pathogenic and economic GIN globally (Coles, 1986).

The benzimidazoles are a commonly used drug group in the treatment of GIN in both humans and livestock (Gilleard and Redman., 2016). The extensive and indiscriminate use of benzimidazoles against GINs of ruminants has been reported globally to contribute to the escalation of AR development (Railch *et al.*, 2013; Gilleard and Redman, 2016; Mphahlele *et al*., 2021). Anthelmintic resistance is the ability of previously susceptible parasite populations to survive otherwise lethal drug doses allowing them to pass on this trait to their progeny (Emery *et al*., 2016). Consequently, benzimidazole has been the readily available and most affordable drug, especially for low-income livestock farming communities worldwide, including Southern Africa (Vetta *et al.*, 2006; Mphahlele, 2021). The high

efficacy of anthelmintic drugs and success in controlling GINs has led to extensive but erroneous application among low-education and subsistence-type farming communities. This misuse has contributed to the development of AR, with its detrimental impact on livestock production becoming inconceivable (Baltrušis *et al*., 2020; Mphahlele *et al.*, 2021). AR has been traditionally detected through drug efficacy tests such as the faecal egg count reduction test (FECRT), the egg hatch assay (EHA), and the larval development test (LDT) (Kotze and Prichard, 2016). The World Association for the Advancement of Veterinary Parasitology (WAAVP) has endorsed *in vivo* and *in vitro* test guidelines for detecting AR of different drug groups in various farm animals (Coles *et al.*, 2006; Kotze and Prichard, 2016). These tests, however effective and yielding a fair measure of drug efficacy and the detection of AR in small ruminants, were considered inherently inconsistent, relatively difficult to interpret, time-consuming, labour-intensive, and relatively costly (Kwa *et al.*, 1995; Kotze *et al.*, 2012). The continued development of AR and its economic impacts led to an extensive search for more sensitive, fast and dependable detection protocols. Molecular assays that accurately detect benzimidazole resistance-associated SNPs in the beta-tubulin isotype-1 gene can also determine the composition and frequency of AR-associated alleles in GINs (Kwa *et al.*, 1995; Silvestre and Cabaret, 2002; Gallidis *et al.*, 2012).

Research initiatives on mutations encoding for AR in the beta-tubulin gene investigating the emergence and spread of drug-resistant parasites were intensified by Seesao *et al.* (2017) and Chaudhry *et al.* (2015). Consequentially, assays such as allele-specific PCR (AS-PCR) and restriction fragment length polymorphism (RFLP) for determining benzimidazole resistance-associated mutations at codons 167 (phenylalanine for tyrosine), 198 (phenylalanine to histidine) and 200 (phenylalanine for tyrosine) of the GIN beta-tubulin gene were developed and proved to be accurate and consistent (Prichard, 2001; Silvestre and Cabaret, 2002; Ghisi *et al.*, 2007; Chaudhry *et al.*, 2015; Kumar and Singh, 2017).

GIN infections and AR development are associated with significant costs in livestock production, including reduced productivity, increase in mortality rates, and eventual need for frequent treatment with drugs. Molecular protocols are growing in parasitological work such as parasite identification, genetic diversity, phylogenetic relationships, and in detection of AR-associated SNPs among GIN populations. The present study was undertaken to determine the presence and intensity of alleles responsible for AR by genotyping the beta-

tubulin isotype-1 gene from pooled L_3 DNA of *Haemonchus contortus* from naturally infected Merino sheep populations in Lesotho. The study also assessed the phylogenetic status, genetic diversity, and population structure of *H. contortus* in Lesotho.

6.2. Materials and Methods

6.2.1. Primer selection of Haemonchus contortus beta-tubulin isotype-1 partial gene of benzimidazole-associated anthelmintic resistance-determination

To determine the variations within the beta-tubulin isotype-1 gene SNPs associated with AR in the benzimidazole drug group, a gene region encompassing codons 167, 198, and 200 was amplified, purified and sequenced. The genomic DNA obtained from morphometrically identified L_3 in Chapter 4 was further subjected to AS-PCR protocol for AR determination. The choice of molecular markers was based on previous benzimidazole resistance work by Kwa *et al.* (1993), Elard *et al.* (1996), and Silvestre and Humbert (2000). Primer selection was based primarily on the successful genotyping and determination of AR-associated SNPs (TTC to TAC) at codon 200 of the beta-tubulin isotype-1 gene of trichostrongylids. Overall, six primers were purchased, and a nested PCR followed by multiplex-type AS-PCR using four primers in one reaction was carried out (Table 6.1). A nested PCR method was used from already established primer sets of the beta-tubulin isotype-1 gene consisting of nine exons and 10 introns. Primer validation for the target gene segment was performed using positively identified *H. contortus* larvae specimens from South Africa and by evaluating the amplicon size on 1.5% agarose gel electrophoresis. The PCR amplification conditions were incorporated as published by Coles *et al.* (2006) and Silvestre and Humbert, (2000) with minor modifications for protocol optimization.

Table 6.1: Beta-tubulin isotype-1 gene oligonucleotide primers used in the nested PCR and AS-PCR in the determination of benzimidazole resistance in *H. contortus* in Lesotho presented with their relative volumes per reaction.

Primer Id	Sequence	Publishers	Quantity in Reaction
PN1	Fw: (5'-GGCAAATATGTCCCACGTGC-3')	Kwa *et al*. (1993)	3 µL
PN2	Rv: (5'GATCAGCATTCAGCTGTCCA3')		3 µL
Ph1	Fw: 5′-GGAACAATGGACTCTGTTCG-3′	Silvestre and	0.5 µL
Ph2	Rv: 5′-GATCAGCATTCAGCTGTCCA-3′	Humbert (2000),	1.0 µL
Ph3	Fw5′-CTGGTAGAGAACACCGATGAAACATA-3′	Coles *et al.*, 2006	1.0 µL
Ph4	Rv: 5′-TACAGAGCTTCGTTGTCAATACAGA-3′		0.5µL

6.2.2. Primary amplification of Haemonchus contortus beta-tubulin isotype-1 partial gene

Oligonucleotide primers used for the initial PCR were PN1 forward primer (5'GGCAAATATGTCCCACGTGC3') and PN2 reverse primer (5'GATCAGCATTCAGCTGTCCA3') as per Silvestre and Humbert (2000) and Coles *et al.* (2006). A 25 µl PCR reaction was comprised of 3 µl of the DNA, 20 pmol of each primer (PN1 and PN2), 12.5 µl of Biorad supermix, and 9.5 µl of DNase-free water. The PCR reaction was such that initial denaturation was at 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 55 seconds, 57 °C for 55 seconds, and 72 °C for 55 seconds. The final extension was 72 °C for 10 minutes.

6.2.3. Nested PCR

To verify that the target gene segment was being amplified, an amplicon from the PN1/PN2 product was taken as a template for the second step (nested) PCR involving the Ph1 and Ph2 primer sets (Table 6.1). A set of non-specific forward Ph1 (5'-GGAACAATGGACTCTGTTCG-3') and reverse non-specific primer Ph2 (5'-GATCAGCATTCAGCTGTCCA-3') were used with each 0.5 µL in a reaction total volume of 25 µL. Supermix and DNase-free water were added as in the primary PCR protocol. The thermocycler conditions were as in the primary PCR protocol. The second PCR yielded a 750 bp segment, which was further amplified using non-specific primers that included exons 3–5 and their intervening introns of the beta-tubulin isotype-1 partial gene, as demonstrated

in Figure 6.1. The resultant partial gene segment also included three codons (167, 198, and 200) of the beta-tubulin isotype-1 gene known to be associated with benzimidazole resistance.

6.2.4. Purification and sequencing of PCR products

For genotyping SNPs responsible for benzimidazole-resistance, L_3 samples identified positive for *H. contortus* by morphometric analysis and by using ITS-2 molecular marker were then subjected to beta-tubulin isotype-1 non-specific primers set flanking 750 bp partial gene The partial gene comprised a segment from exon 3 to exon 5. PCR amplicons were analysed on a 1.5% agarose gel electrophoresis in 1X TAE buffer on a BioRad imaging System. Column purification was employed on distinct single bands. In contrast, the gel purification method was used only when there were multiple bands, from which the target band size was expunged and purified accordingly using Qiagen commercial kits. The purified products were then sent in duplicates of reverse and forward primers to the Sanger sequencing facility in Germany. Raw sequences were blasted on the NCBI platform for homology check and edited using Staden package software, version 2.0.0, and consensus sequences were assembled for downstream bioinformatics analysis (Staden *et al*., 2000).

6.2.5. Genotyping of Haemonchus contortus beta-tubulin isotype-1 gene for determining presence and frequency of anthelmintic-resistance SNP at codon 200.

The specific AS-PCR protocol used in the present study only determined the presence of a mutation on codon 200 of the beta-tubulin isotype-1 gene. The frequencies of gene alleles responsible for benzimidazole AR on codon 200 of the beta-tubulin isotype-1 gene of *H. contortus* L_3 were also determined. An amplicon from the PN1/PN2 product was then used as a template in a second-step (nested) PCR involving the Ph1 and Ph2 primer sets (Table 6.1**).** The segment also encompasses three common codons said to be responsible for AR.

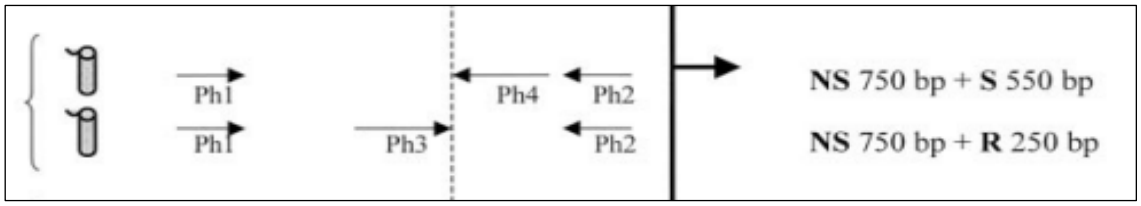

Figure 6.1: AS-PCR for determination of point mutation on the beta-tubulin isotype-1 partial gene for benzimidazole resistance on L3 of *H. contortus*. where Ph1 is non-specific forward, Ph2 is non-specific reverse, Ph3 is a resistant specific forward primer and Ph4 is a susceptible specific reverse primer. NS is a nonspecific fragment, S is a susceptible-specific gene fragment; R is a benzimidazole resistance-specific gene fragment (adapted from Silvestre and Humbert, 2000)

The genotypes of *H. contortus* in adult worms were studied using allele-specific PCR-based testing (Kwa *et al.,* 1994). The present study considered codon 200, a common locus for a benzimidazole resistance-associated SNP. To target the SNP at codon 200 (TTC/TAC) of the beta-tubulin isotype-1 gene of *H. contortus,* four different primers (Ph1, Ph4, Ph2, and Ph3) were used in one PCR mix. Ph1 and Ph2 were each 0.5 µl, while the allele-specific primers Ph3 and Ph4 were 1 µl each. This is a competitive AS-PCR comprising two non-specific primers forward and reverse of Ph1 (5'-GGAACAATGGACTCTGTTCG-3') and reverse non-specific primer Ph2 (5'-GATCAGCATTCAGCTGTCCA-3') with allele-specific susceptible primer Ph4 (5'-TACAGAGCTTCGTTGTCAATACAGA-3) and a specific allele resistant primer Ph3 (5'-CTGGTAGAGAACACCGATGAAACATA-3'). These four primer sets were designed to amplify three different beta-tubulin gene fragments. (1) Primers Ph1 and Ph2 are universal non-specific and yield a non- benzimidazole-resistance-specific fragment (750 bp) of the gene. (2) The presence of a benzimidazole-susceptible specific reverse primer in a three-primer set (Ph1, Ph2, and Ph3) is responsible for the 550 bp gene segment, which is symbolic of the presence of a susceptible allele (S). (3) A primer set involving a benzimidazole resistant-specific forward primer with a universal reverse primer (Ph1/Ph2/Ph4) yields a 250 bp gene segment that represents the resistant allele (R) on codon 200 of the beta-tubulin isotype-1 gene. von Samson-Himmelstjerna (2006) formulated a protocol in which all four primers can be used simultaneously in one reaction, taking advantage of different fragment sizes as in Figure 6.1. Table 6.1 presents the

sets of primers used and their respective volumes in the AS-PCR protocol for a 25 µl PCR. The unclear results from the four-primer protocol were repeated by running two separate AS-PCRs of allele-specific primers divided into separate reactions with the non-specific primers. The PCR was such that the reagents comprised 12.5 µl BioRad supermix, 2 µl template, 0.5 µl primer volumes, and DNA-free water was added to make a total reaction mix of 25 µl. The thermocycler was set as per Silverstre and Humbert (2000), with some minor modifications. The final thermocycler reaction was such that the initial denaturation was at 94 °C for 5 minutes, followed by 40 cycles of 94 °C denaturation for 55 seconds, 57 °C annealing for 55 seconds, 72 °C extension for 55 seconds, and the final extension was 72 °C for 10 minutes. Amplicons produced from all PCRs were viewed and analysed on a 1.5% electrophoresis gel.

6.2.6. Data analysis and statistical inferences

The NCBI's nucleotide Blast programme (http://www.NCBI.nlm.gov/BLAST) was used to align and analyse sequences to identify the species and level of homology with global sequences in the gene bank. The consensus sequences were assembled and edited in MEGA11 software (Tamura *et al.*, 2021). To evaluate the genetic relationship between sequences, phylogenetic tree analysis with bootstrap tests and pairwise alignment of estimates of evolutionary divergence were analysed in MEGA11 software (Tamura *et al.*, 2021). The aligned sequences were then converted to Nexus/Paup format and transferred to the DNAsp version for further downstream analysis. The DNAsp platform was used to infer molecular indices for populations' genetic analyses. Descriptive statistics were used to collate and analyse data on AS-PCR and allele frequency determination. The chi-square test (χ^2) to determine the existence statistical relation between resistance-associated alleles and Lesotho's topographic regions.

6.3. Results

6.3.1. Amplification and genotyping of beta-tubulin isotype-1 gene for susceptible and resistant SNPs at codon 200

The nested PCR was successfully executed for the beta-tubulin isotype-1 partial gene of *H. contortus* from naturally infected Merino sheep. The amplified partial gene of ≈750 bp

produced in the current study is presented in Figure 6.2. DNA from five pooled L_3s from naturally infected Merino sheep was subjected to nested PCR. The four-primer set AS-PCR protocol was used to determine different benzimidazole resistance-associated alleles from different ecological zones of Lesotho. There were 89 *H. contortus* L_3 suspension samples genotyped by AS-PCR revealing the presence of benzimidazole-resistant-associated alleles from *H. contortus* populations across four ecological zones in Lesotho. The AS-PCR of the beta-tubulin isotype-1 partial gene revealed three DNA band patterns: (1) a non-specific gene segment of 750 bp; (2) a susceptible allele-specific gene segment of 550 bp; and (3) a resistant allele-specific gene segment of 250 bp (Figure 6.3). The three genotypes recorded were homozygous susceptible (SS), heterozygous (SR), and homozygous resistant (RR), with overall percentage frequencies of 51.7%, 32.6%, and 15.7%, respectively (Table 6.2). The average phenotypic frequencies for susceptible (S-) and resistant (RR) strains were 84.3 and 15.7%, respectively (Figure 6.4). The highest susceptibility (91%) to benzimidazole was recorded in the Lowlands, while the highest resistance frequency (24%) in the study was recorded in the Highlands. Notwithstanding, the Pearson Chi-square test revealed no significant association between benzimidazole-associated alleles and ecological zones (χ^2 = 4.216, df = 6, p = 0.647).

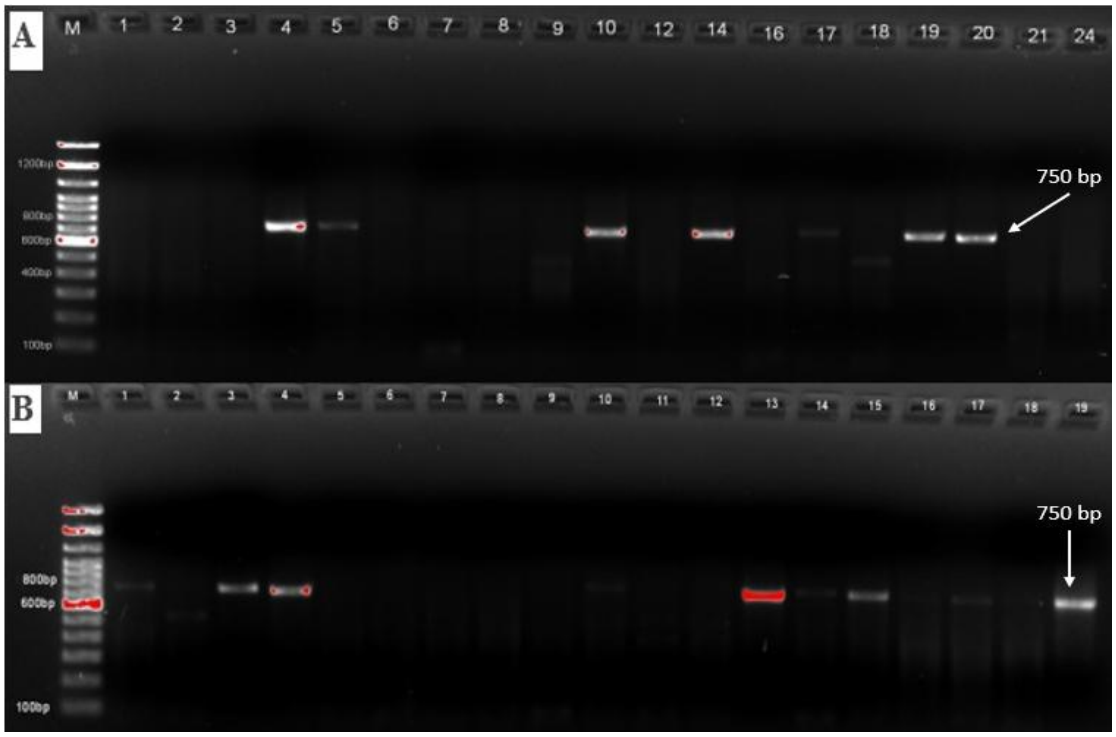

Figure 6.2: The image of agarose gel electrophoresis of *H. contortus* larvae amplicons showing beta-tubulin isotype-1 partial gene PCR product of ±750 bp. 1.5% agarose gel was run on 100 volts for 50 minutes. DNA ladder of 100 bp was used. Figure 6.2A. 1-5 Lowlands Collection Sites, 6-10 Foothills, 12, 14, 16, and 18 from Lowlands, and 21, and 24 were SRV on the gel A. Figure 6.2B 1-5 were from Highlands, 10-14 from Lowlands and 15-19 were from SRV.

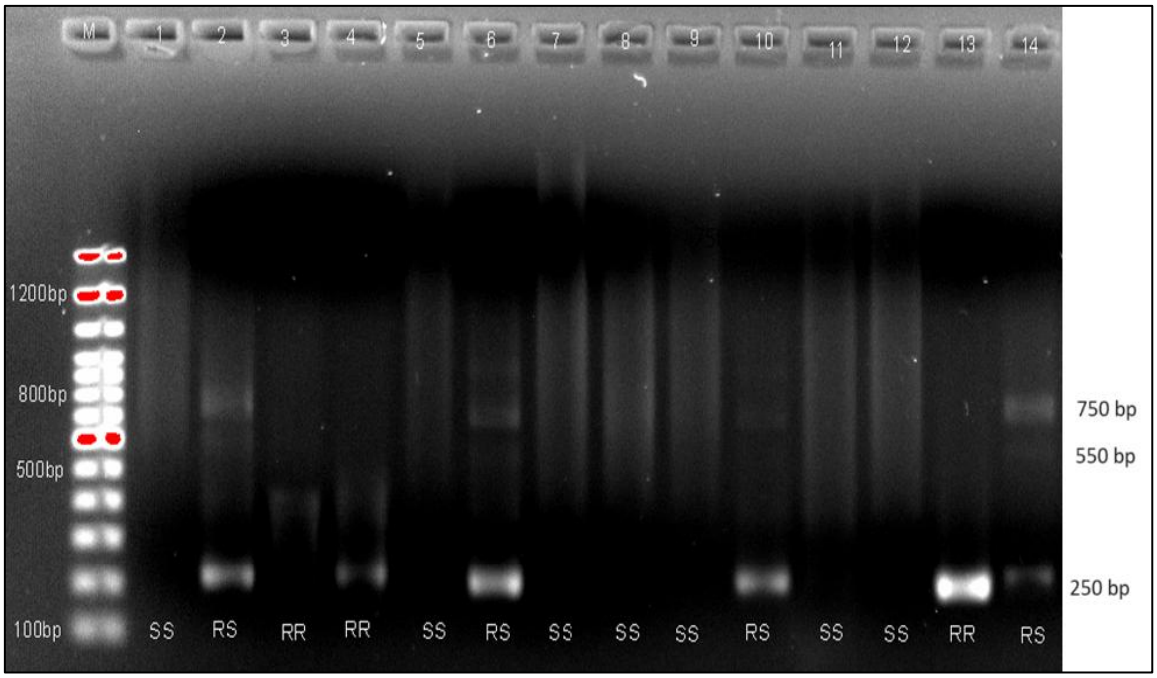

Figure 6.3: Allele Specific PCR gel electrophoresis pictures from four primer set reaction of beta-tubulin partial gene showing Lanes with M 100 bp DNA ladder, SS: Homozygous Susceptible isolate (750 bp) (Lanes 1, 5, 7, 8, 9, 11, 12), SR: (750 bp, 550 bp, and 250 bp) Heterozygous isolate (Lanes 2, 6, 10, 14), RR: Homozygous Resistant isolate (250 bp) (Lanes 3, 4, 13).

Table 6.2: Allele-specific PCR genotyping benzimidazole resistant and susceptible isolates of beta-tubulin isotype-1 gene of *H. contortus* L_3 from Merino sheep. The table presents the frequency (%) of the codon 200 variants.

Ecological Zone	N	Genotypic frequency (mean %) no. of samples			Phenotypic frequency (mean %) no. of samples		Statistics
		Homozygous Susceptible (SS)	Heterozygous (SR)	Homozygous Resistant (RR)	Susceptible (S-)	Resistant (RR)	
Highlands	25	(10) 40	(9)36	(6) 24	76 (19)	24 (6)	
Foothills	21	(13) 62	(5)24	(3) 14	86 (18)	14 (3)	$\chi 2 = 4.21$
Lowlands	26	(15) 58	(9)35	(2) 8	91 (24)	9 (2)	df = 6
SRV	17	(8) 47	(6) 35	(3) 18	85 (14)	15 (3)	p = 0.647
Total	**89**	**(46) 51.7**	**(29) 32.6**	**(14) 15.7**	**84.3**	**15.7**	

N = total number of samples tested

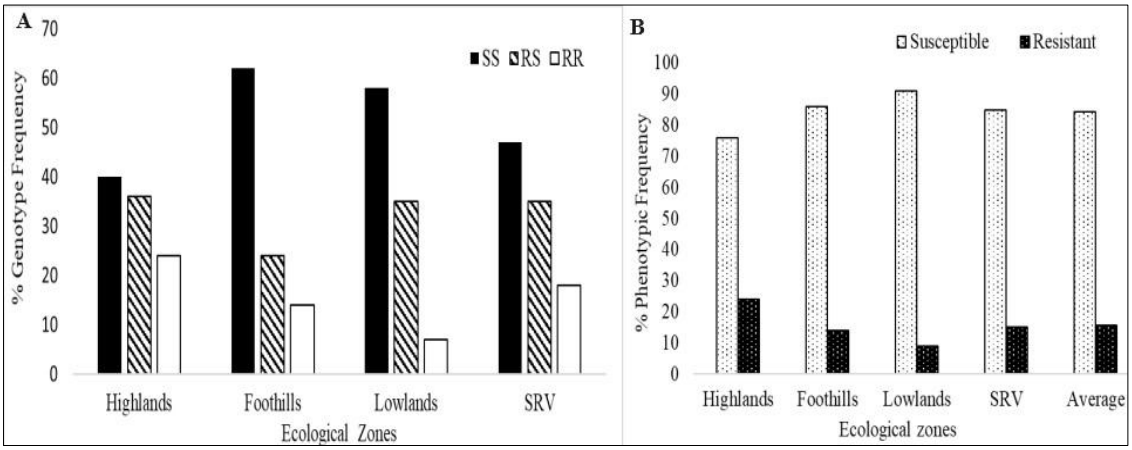

Figure 6.4: The percentage frequency of genotypes and phenotypes of alleles associated with benzimidazole resistance in codon 200 of beta-tubulin isotype-1 of *H. contortus*. (A). Genotypic frequencies of alleles from four ecological zones of Lesotho (SS=Homozygous susceptible, SR = Heterozygous, RR = Homozygous resistant), (B). Phenotypic frequencies depicting susceptibility and resistance in beta-tubulin isotype-1 of *H. contortus* isolates from four ecological zones in Lesotho.

6.3.2. Genotyping benzimidazole resistance-associated SNPs on codon 200 of beta-tubulin isotype-1 partial gene of Haemonchus contortus from four ecological zones of Lesotho

Upon running a sequence pairwise comparison of Lesotho *H. contortus* isolates with beta-tubulin isotype-1 partial genes from the GenBank, a homology between 96 and 100% was recorded. The beta-tubulin isotype-1 gene, accession number X67489 was used as a reference. Multiple pairwise alignment revealed estimates of evolutionary divergence on the beta-tubulin isotype-1 partial gene among Lesotho *H. contortus* isolates ranging between 0.00160 to 0.03595, from BT_LL_13 vs BT_SRV_24 and BT_LL_12 vs BT_HL_04 respectively (Table. 6.4).

Genotyping for benzimidazole resistance or susceptibility by AS-PCR was further confirmed by Sanger sequencing of the beta-tubulin isotype-1 partial gene. An electropherogram showing codon sections of the beta-tubulin isotype-1 gene associated with AR in *H. contortus* at loci 167, 198, and 200 is shown in Figure 6.5. Sequences of the beta-tubulin isotype-1 partial gene displayed only two types of polymorphisms in codon 200, characteristic of both the resistant and susceptible genotypes (TTC and TAC). All seven sequences produced in the study revealed a susceptible genotype in codon 167 (Figure 6.5A) and GAA in codon 198 (Figure 6.5B). Alternatively, two different genotypes were exhibited on codon 200, depicting the susceptible (TTC) and the resistant mutants (TAC) (Figure 6.5C). In the seven sequenced beta-tubulin isotype-1 partial genes, which included codon 167 or 198, no mutant variations suggesting SNPs for benzimidazole resistance were observed.

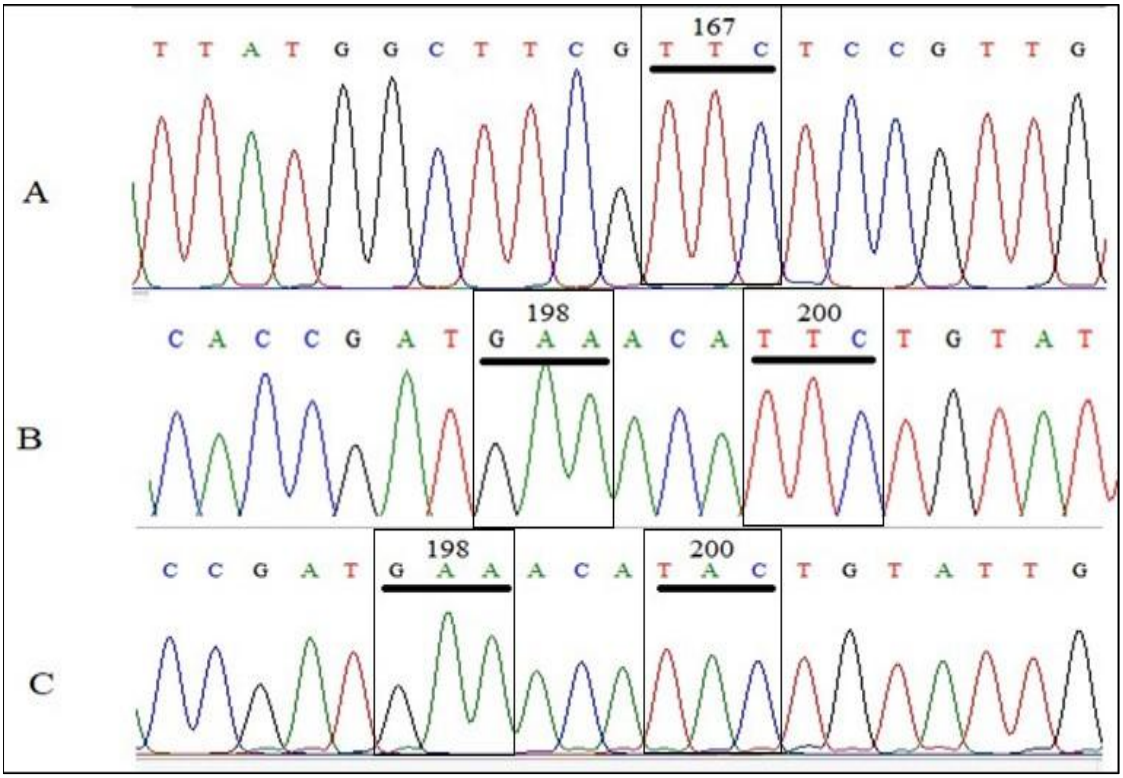

Figure 6.5: The section of PCR products of beta-tubulin isotype-1 partial gene depicting nucleotides at three different SNP loci associated with benzimidazole resistance. **A** shows codon 167 with TTC benzimidazole-susceptible. **B** demonstrates codons 198 and 200 with GAA and TTC respectively both benzimidazole susceptible genotypes. **C** represents codons 198 GAA (benzimidazole-susceptible) and 200 with TAC (benzimidazole-resistant mutant genotype).

The sequenced beta-tubulin isotype-1 partial genes yielded segments ranging from 650 to 690 bp. After editing and trimming the resultant consensus sequences were 626 bp. The sequences were then subjected to multiple sequence alignments and bioinformatic analysis. There was a total of 626 total nucleotide sites, including a gap due to an insertion mutation. The multiple alignment of sequences in the present study resulted in 586 monomorphic sites and 40 polymorphic sites. Polymorphic sites comprised 17 transversions (three G < > T, five T < > A, six A < > C, and three G < > C) and 23 transitions (12 T < > C and 11 A < > G). The beta-tubulin isotype-1 partial gene sequences in the present study resulted in 20 singleton variable sites, each with two variants (87, 173, 194, 258, 273, 379, 381, 397, 419, 420, 422, 437, 449, 466, 469, 503, 511, 624) and three variants (169, 403). There were 20 parsimony informative sites, each with two variants (99, 139, 140, 158, 167, 181, 191, 349,

352, 390, 393, 399, 404, 405, 411, 429, 432, 528, 609, 619) and three variants (88). There was a single insertion mutation between the nucleotide positions 394 and 395 of adenine in all Lesotho isolates. The nucleotide polymorphic sites of beta-tubulin isotype-1 sequences representing intra-species variations are presented in Table 6.3. A GC content of 49% was recorded for the *H. contortus* sequences. Seven amplicons were successfully amplified, purified and sequenced on both forward and reverse duplicates. The consensus sequences comprised five susceptible (TTC) and two resistant genotypes (TAC) in codon 200 (Table 6.3). Figure 6.6 demonstrates multiple sequence alignments of the beta-tubulin isotype-1 partial gene of *H. contortus*. The sequences had polymorphic sites spanning regions from exon 3 to exon 5 and their associated introns. The sequenced partial gene also included SNPs responsible for benzimidazole resistance (codons 167, 198, and 200). Pairwise estimates of the evolutionary divergence of seven beta-tubulin isotype-1 partial gene sequences from four ecological zones of Lesotho derived from MEGA 11 are presented in Table 6.4. The pairwise evolutionary divergence estimates between Lesotho isolates had the lowest variability (0.00160) between isolates BT_LL_13 from the Lowlands and BT_SRV_24 from the Senqu River Valley. Alternatively, the highest pairwise divergence value (0.03595) came between sequences BT_HL_04 and BT_LL_12 from the Highlands and Lowlands, respectively.

Table 6.3. Summary of nucleotide polymorphic information depicting intra-species variations of seven beta-tubulin isotype-1 of *H. contortus* sequence isolates from four ecological zones of Lesotho. Genbank sequence accession number X67489 was used as a reference. A dash represents an identical nucleotide with reference.

Nucleotide Variable Positions of beta-tubulin isotype-1 partial gene																				
Isolates	**87**	**88**	**99**	**139**	**140**	**167**	**169**	**173**	**181**	**191**	**194**	**258**	**273**	**349**	**352**	**379**	**381**	**390**	**396**	**398**
X67489	A	T	C	A	C	T	G	T	C	C	G	A	G	G	T	C	T	G	C	A
HL_BT_01	-	-	-	C	G	-	T	A	A	-	A	T	-	C	C	T	-	-	T	G
HL_BT_04	C	A	A	C	G	C	T	A	A	T	A	T	-	C	C	T	-	-	T	-
FH_BT_10	-	-	-	-	-	-	A	A	-	-	A	T	-	C	C	T	G	-	T	G
LL_BT_12	-	-	-	C	-	C	T	A	-	-	A	T	A	-	-	T	-	C	T	-
LL_BT_13	-	C	A	C	G	C	T	A	A	T	A	T	-	C	C	T	-	C	T	-
SRV_BT_20	-	C	A	C	G	C	T	A	A	T	A	T	-	C	C	T	-	-	T	G
SRV_BT_24	-	C	A	C	G	C	T	A	A	T	A	T	-	C	C	T	-	C	T	-
Nucleotide Variable Positions of beta-tubulin isotype-1 partial gene																				
	403	**404**	**405**	**411**	**419**	**420**	**422**	**429**	**432**	**437**	**449**	**464**	**469**	**504**	**511**	**528**	**609**	**619**	**624**	**403**
X67489	T	G	A	G	A	C	T	A	T	G	A	C	A	C	G	T	A	T	A	T
HL_BT_01	C	T	T	-	-	-	-	G	C	-	-	T	G	T	A	-	-	A	G	C
HL_BT_04	A	-	-	-	-	T	-	-	-	-	-	T	G	T	A	-	-	A	G	A
FH_BT_10	C	T	T	-	-	-	-	G	C	-	-	T	G	T	A	-	-	A	G	C
LL_BT_12	C	T	T	-	C	-	C	G	C	A	T	T	G	T	A	-	C	-	G	C
LL_BT_13	C	T	T	A	-	-	-	G	C	-	-	T	G	T	A	A	C	-	G	C
SRV_BT_20	C	T	T		-	-	-	G	C	-	-	T	G	T	A	-	-	A	G	C
SRV_BT_24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-

Table 6.4: Estimates of evolutionary divergence between beta-tubulin isotype-1 partial gene sequences from four ecological zones in Lesotho. The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Tamura 3-parameter model (Tamura, 1992). This analysis involved seven nucleotide sequences All positions containing gaps and missing data were eliminated. There was a total of 626 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*., 2021)

Isolates	**EZ**	**BT_HL_01**	**BT_HL_04**	**BT_FH_10**	**BT_LL_12**	**BT_LL_13**	**BT_SRV_20**	**BT_SRV_24**
BT_HL_01	Highlands							
BT_HL_04	Highlands	0.02104						
BT_FH_10	Foothills	0.00802	0.02928					
BT_LL_12	Lowlands	0.02433	**0.03595**	0.02597				
BT_LL_13	Lowlands	0.01777	0.02103	0.02597	0.02268			
BT_SRV_20	SRV	0.00641	0.01613	0.01449	0.02763	0.01125		
BT_SRV_24	SRV	0.01614	0.01939	0.02432	0.02433	**0.00160**	0.00963	

6.3.3. Genetic diversity and phylogenetic analysis of beta-tubulin isotype-1 partial gene in Haemonchus contortus from Lesotho

The beta-tubulin isotype-1 gene is a molecular marker used mainly for evaluating the presence of AR, assessing genetic diversity, and mapping gene flow among GIN populations. Haplotype diversity is calculated as a measure of the variation and distribution of alleles in a population. DNAsp version 6.12.03 package was used to evaluate Haplotype diversity, an index used to estimate the probability of at least two randomly chosen haplotypes being different. In the current study, a high level of haplotype diversity of 1.000 ± 0.076 and nucleotide diversity (π) of 0.01891 were used to assess the level of diversity among the beta-tubulin isotype-1 partial gene sequences (Table 6.5.). A negative Tajima's D of -0.35927 was statistically insignificant at $p > 0.10$, and Fu's Fs of -1.266 were recorded for seven beta-tubulin isotype-1 partial genes of *H. contortus* in the present study.

The phylogenetic trees were constructed in MEGA11 (Tamura *et al*., 2021), to investigate the association of 26 sequences of partial beta-tubulin isotype-1 from *H. contortus* isolates from Lesotho (n=7 De novo) and other countries (n=18 retrieved from the GenBank) (Table 6.6, Fig. 6.7A andB). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown beside the branches (Felsenstein, 1985). The tree was rooted using another Trichostrongyloid nematode of ruminants, *Teladosargia circumcinta* (accession number KF483619). The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992) and presented in units of base substitutions per site. The phylogenetic tree showed a common ancestry among *H. contortus* isolates from Lesotho and other global sequences and distinct clustering patterns depicting three major clades (A, B, and C). The outlook and structure of the Maximum Likelihood Method (Fig. 6.7A) and the Neighbour-Joining Method (Fig. 6.7B) constructed phylogenetic tree algorithms were generally similar. All seven Lesotho sequences clustered in Clade-A together with six other global sequences from different countries. Clades-B and Clade-C included sequences from different countries without any apparent geographic pattern.

Figure 6.6: Multiple sequence alignment of *H. contortus* beta-tubulin isotype-1 (626 bp) from Lesotho using accession number X67489 (resistant strain) as reference. The segment corresponds to the partial gene starting from part of exon 3 to exon 5 with associated intervening introns. Codon 200 is highlighted with two TAC and five TTC SNPs.

Table 6.5: Genetic diversity indices of seven beta-tubulin isotype-1 partial gene sequences of *H. contortus* L_3 from Merino sheep from four ecological zones of Lesotho.

	N	NPS	H	SS	Hd	Pi (π)	T's D	Fu's Fs
Partial beta-tubulin gene	7	30	7	41	1.000 ± 0.076	0.01891	-0.35927	-1.266

N = Number of sequences, NPS = Number of polymorphic sites, H = Number of haplotypes, SS = sites with substitutions, Hd = Haplotype Diversity, Pi (π) = Nucleotide diversity, T'sD = Tajima's D value, Statistical significance: Not significant, P > 0.10, Fu's Fs = Fu's Fs neutrality test

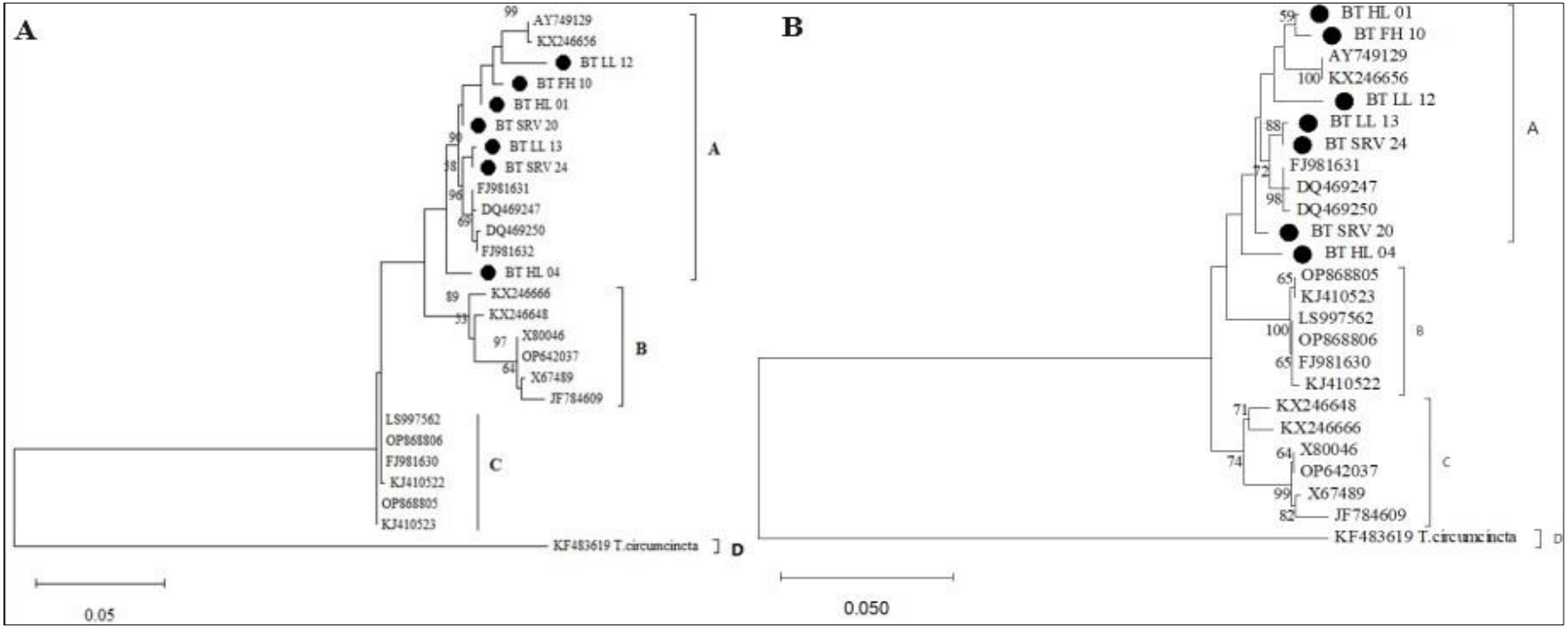

Figure 6.7: Phylogenetic tree analysis showing an evolutionary relationship between the beta-tubulin isotype-1 partial of *H. contortus* isolates from Lesotho and those retrieved from the GenBank. Lesotho isolates are distinctively shown with a black dot, while other global isolates are presented with their NCBI accession numbers. This analysis involved 26 nucleotide sequences. Figure 6.7A. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura 3-parameter model (Tamura 1992). Figure 6.7 B. The evolutionary history was inferred using the neighbour-joining method (Saitou. and Nei 1987).

Table 6.6. presents population pairwise fixation index (F_{ST}) values beta-tubulin isotype-1 gene from three ecological zones in the study. The highest genetic differentiation (F_{ST} = 0.11475) was recorded between the Highlands and Lowlands while the lowest was (F_{ST} = 0.0000) between the Lowlands and the SRV. Figure 6.8. demonstrates a distance-based median-joining haplotype analysis and an unrooted phylogeographic comparison used to differentiate Lesotho *H. contortus* isolates from those in the GenBank.

Median-joining haplotype analysis is a distance-based and unrooted phylogeographic comparison used to compare Lesotho *H. contortus* isolates with those from the GenBank, as demonstrated in Fig. 6.8. Median-joining haplotype analysis of 25 beta-tubulin isotype-1 partial gene sequences were used in the present to infer haplotype networks, evolutionary relationships, and conceptualize population structure based on the beta-tubulin isotype-1 partial gene among *H. contortus* isolates from Lesotho and with those from several countries around the globe. 25 beta-tubulin isotype-1 partial gene sequences were aligned by a 626 bp segment and analysed by median-joining haplotype analysis. The network analysis revealed analysis resulted in 19 distinct haplotypes. Isolates from Lesotho had seven sequences, all of which became distinctive haplotypes. Consequently, none of the isolates from Lesotho's beta-tubulin isotype-1 shared haplotypes with others from around the globe. Notwithstanding, Hap_7 (SRV_BT_24) and Hap_9 (LL_BT_13) were the closest since they only had a single mutation difference between them. This also showed the uniqueness of Hap_6 (HL_BT_04) from the Highlands ecological zone exhibited the most genetically distant variant among Lesotho isolates due to having more mutations as presented in neighbour-joining and maximum likelihood phylogenetic trees. The European isolates, Hap_10, 11, and 12, from Turkey, Italy, the UK, and Switzerland, also portray close haplotypic relationships, with two mutations amongst them. Table 6.6: Population Pairwise F_{ST} values of beta-tubulin isotype-1 gene of *H. contortus* four ecological zones of Lesotho.

Table 6.6: Population Pairwise F_{ST} values of *H. contortus* L_3 from three ecological zones of Lesotho based on beta-tubulin isotype-1 gene

Ecological Zone	Highlands	Lowlands	Senqu River Valley
Highlands	-	-	-
Lowlands	0.11475	-	-
Senqu River Valley	- 0.05556	0.00000	-

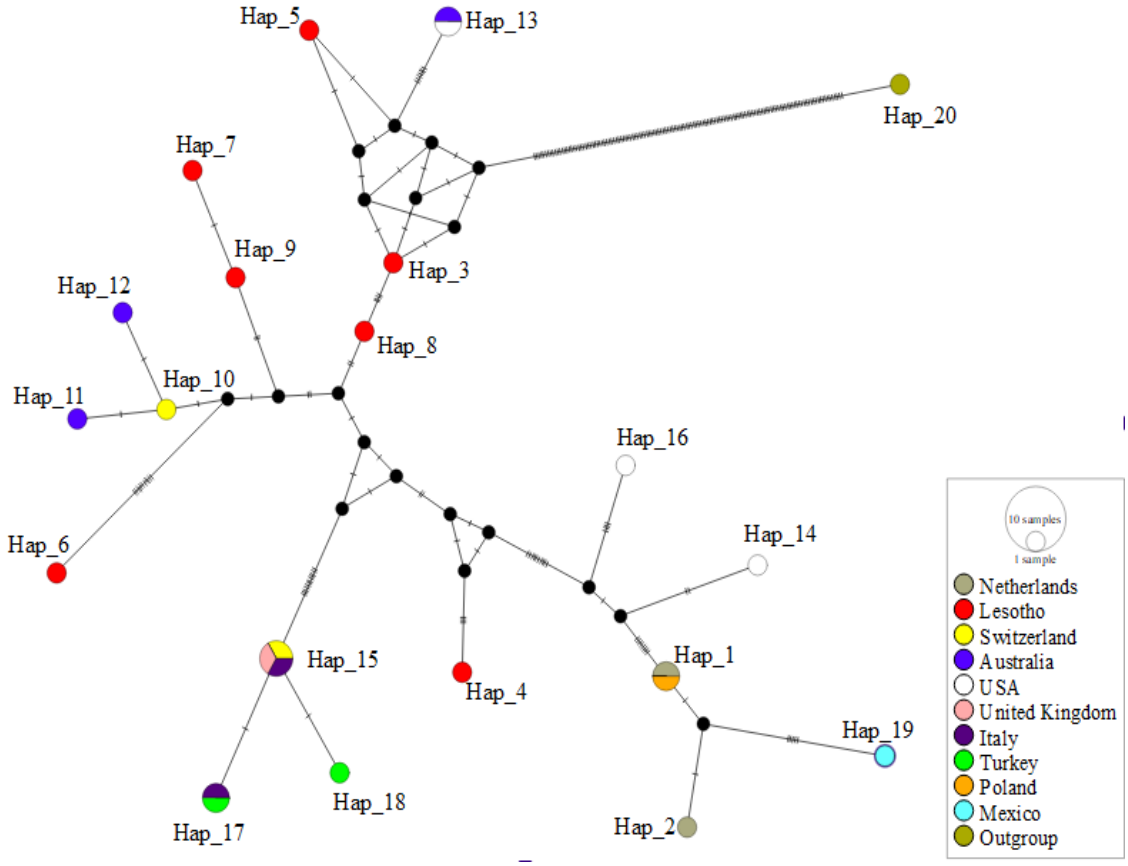

Figure 6.8: Median-joining haplotype network analysis of *H. contortus* beta-tubulin isotype-1 partial gene (626 bp length) of Lesotho isolates and selected global sequences. 19 haplotypes resulted from an overall alignment of 25 beta-tubulin sequences. Haplotype number 20 is the *T. circumcinta*. In the analysis, each disc represents a haplotype; the disc size depicts the relative frequencies for each haplotype. Different colour shades represent

specified countries. Mutation changes are represented by lines between the discs. Black dots are median vectors (Bandelt *et al*. 1999).

6.4. Discussion

The present study genotyped the beta-tubulin isotype-1 gene to diagnose the presence of susceptible and resistant alleles associated with benzimidazole resistance in *H. contortus* populations isolated from naturally infected Merino sheep in Lesotho. Cellular and genetic mechanisms of benzimidazole resistance revealed that a SNP in the gene encoding beta-tubulin isotype-1 is responsible for such mutations (Kwa *et al*., 1993). Benzimidazole drugs are bound to the nematode tubulin target, consequently interfering with the normal functioning of microtubules (Robinson *et al*., 2004). Sangster *et al*. (1985) reported a reduction of microtubules in the intestinal cells of susceptible GIN post-benzimidazole treatments and a contrasting result in resistant parasites. Microtubules are cellular structures that facilitate cell division and intracellular transport and weakening them interferes with intracellular transport and nutrient absorption, ultimately hindering cell division processes (Lacey, 1988; Straight and Field, 2000). Čudeková *et al*. (2010) investigated AR using phenotypic and genotypic protocols on *H. contortus* and established a positive correlation between EHT and AS-PCR methods; the study concluded that it was more expeditious to use molecular genotyping. Furthermore, von Samson-Himmelstjerna *et al*. (2006) also reported that molecular methods for AR detection, such as AS-PCR and pyrosequencing, were able to detect early stages of AR, while EHT had about a 30% threshold of alleles before it could yield any credible results. Allele-specific PCR tools were deemed cheaper and faster than the labour-intensive and time-consuming conventional methods for AR detection in GINs. Moreover, molecular-based protocols were also more convenient for monitoring resistance in field situations and, as a result, could be adapted for wider AR surveys (Irum *et al*., 2014; Knapp-Lawitzke *et al*., 2015). Chandra *et al*. (2014) reported that AS-PCR was a reasonably quicker and more reliable protocol to determine benzimidazole resistance even during the initial stages of AR development. Lambert *et al*. (2017) also reiterated that the protocol may be sensitive enough to evaluate either susceptibility or resistance to the benzimidazole group for field isolates, even where the drugs may not have been used. However, its accuracy depends on several factors and, as such, should be used in conjunction with other methods and validated against known resistant and susceptible

samples to ensure reliable results. Additionally, ongoing surveillance and monitoring of resistance are essential due to the dynamic nature of parasite populations and the potential for evolving resistance mechanisms. Notwithstanding, the contentment of some scholars in using both conventional and molecular-based AR tests simultaneously due to yielding a considerable correlation was granted. Rialch *et al*. (2013), however, cautioned the purported correlation was not always yielding dependable results due to the influence of factors such as the variations in immunology and pharmacokinetics of different host animals. Genotyping of the beta-tubulin isotype-1 gene for benzimidazole resistance-associated alleles presence in codon 200 was performed as the first AR determination among *H. contortus* populations in naturally infected Merino sheep in Lesotho. Genotyping codon 200 of the beta-tubulin isotype-1 gene was chosen due to literature reports of it being the most prevalent among its counterparts (codons 167 and 198) (Silvestre and Humbert, 2000; Garg and Yadav, 2009; Mohanraj *et al*., 2017; Munguia *et al*., 2018).

AS-PCR was used to detect susceptible (TTC) or resistant mutant genotypes (TAC) in *H. contortus* L_3 beta-tubulin isotype-1 gene codon 200 (Kwa *et al.*, 1994; Elard *et al.*, 1996). The AS-PCR protocol used was described by Silvestre and Humbert (2000) in determining resistance-associated point mutations using adult *H. contortus*. Mohanraj *et al.* (2017) conducted a similar experiment on organised sheep farms using L_3. The pooled DNA used yielded good estimates of SNP frequencies for different alleles in a population. Munguia *et al.* (2018) advanced the sequencing of pooled DNA from adult *H. contortus* and permitted a clear evaluation of benzimidazole-associated SNPs. A similar approach was employed, even though in the present work L_3 DNA was used instead of adults to successfully detect the benzimidazole-responsible SNPs at codon 200 by AS-PCR. The AS-PCR results revealed the presence of three types of benzimidazole-resistance-associated alleles: homozygous resistant (RR), homozygous susceptible (SS), and heterozygous (SR). The overall allele frequency in the study area was 51.7%, 32.6%, and 15.7% for homozygous susceptible, heterozygous, and homozygous resistant, respectively. The three sets of alleles were present in all four ecological zones, although at varying frequencies. A similar composition of alleles (RR, RS, and SS) recorded in our results was also observed by Tiwali et. al. (2007). Salle *et al* 2017 results had comparable trends in terms of alleles composition, also recording significant frequencies of heterozygous genotypes. However, the results of the present study were not statistically different ($P > 0.05$) across all four ecological zones. Similar to our

results, Onder *et al.* (2016) recorded point mutations (TTC to TAC) in codon 200, and no mutations in codons 167 and 198 in Turkey. Moreover, the result of the present study differed from those of Mohanraj *et al.* (2017), where there were no homozygous susceptible (SS) but heterozygous (SR) and homozygous resistant (RR) were present. A similar pattern of results was observed in Atanásio-Nhacumbe *et al.*'s (2019) use of real-time PCR for identification of AR-associated alleles with homozygous resistance (RR = 18.8%), homozygous susceptible (SS = 47.8%), and heterozygous (SR = 33.4%) on codon 200 of the beta-tubulin isotype-1 of *H. contortus* in Mozambique.

The Foothills had the highest homozygous susceptible genotype, while the Highlands were high in resistant genotypes (RR = 24%). The results may portray the Highlands as the high-risk zone for benzimidazole AR resistance, while the lowlands are the opposite. The disparity between these two regions may be brought about by different management regimes, mainly due to animal host movement. Highlands have areas that may be regarded as no-man's lands, where there is a high aggregation of animals with a low probability of resting pastures. Furthermore, the Highlands transhumance, low veterinary facilities, and a poor husbandry regime are factors contributing to the relatively high resistance status recorded in Lesotho. Ali *et al.* (2019) demonstrated that the spread of AR-carrying codon 200 (TAC) came from a single source and migrated through the region of Punjab in India. It was apparent that animal migration may be responsible for spreading resistant alleles in ruminants. Intensive transhumance practices and relatively high animal stocking rates in the Highlands of Lesotho are probable determinants of marked parasite intensity and the high occurrence of alleles responsible for AR between the two ecological zones. Notwithstanding several other reasons for higher resistant allele frequency in the Highlands, transhumance could be one of the driving factors for inferring higher gene flow than in the rest of the country. Our results conformed with previous tests, which showed the point mutation in codon 200 (Phy to Tyr) was the most common across the globe (Coles *et al.*, 2006; Onder *et al.*, 2016; Pitaksakulrat *et al.*, 2021). Brasil *et al.* (2012) documented the mutant SNP (TAC) in codon 200 as being the most prevalent, and no mutant in codon 198 was observed in their entire study in Brazil. Alternatively, Zhang (2016) recorded that the homozygous resistant genotype at codon 198 was the most prevalent among *H. contortus* populations in China, which placed F200Y as second while there was no F167Y mutation. Notwithstanding that AS-PCR still produces credible results as in the present study, Morrison *et al.* (2022) argued that genotyping single

larva had relatively better accuracy than sequencing pooled larval populations because single larva provides the actual genotypes rather than the pooled larvae, which yields average estimates. Alternatively, Munguia *et al.* (2018) and Von Samson-Heisenbergern *et al.* (2007) resolved that pooled L_3 SNP genotyping in codons 167 and 200 yielded corresponding results with genotyping the similar populations using both single nematodes and pooled isolate suspensions using the pyrosequencing protocol.

The heterozygosity result among the *H. contortus* isolates in the current study suggested a high possibility of benzimidazole drugs being administered to Merino sheep for extended periods in Lesotho. Parasite populations with high levels of heterozygosity invariably exhibit high genetic variation, which can contribute to their ability to respond favourably to environmental pressures, including resistance development or consequently avoiding the negative effects of inbreeding (Zhang *et al.,* 2016). Chandra *et al.* (2014) discovered there was an increase in resistance alleles and a decrease in susceptible populations after prolonged use of the benzimidazole drug group on organised farms is indicative of the effect of drug administration on parasite genetic profiles. Moreover, Brasil *et al.* (2012) observed all farms that routinely applied benzimidazole consistently for five years in treating sheep against GIN with SNPs associated with benzimidazole resistance in Brazil. This report is in congruency with our discovery that farmers reported an increase in the frequency of anthelmintic treatment in five years in which the benzimidazole group was a predominant remedy. The variation in distribution and frequencies of benzimidazole resistance-associated alleles in the four ecological zones of Lesotho may therefore prove the presence of a variety of factors, including parasite biotic potential, host populations and movements, husbandry regimes, anthelmintic control strategies, animal nutrition, and range management strategies. Zhang *et al.* (2016) suggested heterozygosity was associated with the gradual emergence and development of benzimidazole drugs. Barerre *et al.* (2013) contested that there is a relationship between increased albendazole systemic exposure and changes observed in SNPs associated with AR in the beta-tubulin isotype-1 gene of *H. contortus*.

AS-PCR genotyping of *H. contortus* L_3 for benzimidazole resistance involves identifying specific genetic variations in the parasite associated with resistance to benzimidazole anthelmintic drugs. Heterozygous alleles (SR, 32.6%) and homozygous resistant (RR, 15.7%) isolates recorded in the present study within the *H. contortus* population portray a

relatively significant proportion of resistance alleles. Garg and Yadav (2009) suggested that the presence of such alleles has the potential to escalate benzimidazole resistance, especially if AR-responsible drugs continue to be used carelessly, especially in areas where there is also a lack of a broader strategic parasite control program. To exemplify the consequences of the negative impact of erroneous dosing habits, Leathwick and Luo's (2017) tests revealed that reductions in dose intensity and increased variation in dose administration had a higher selection for AR; hence, there were increased resistance gene frequencies post-corresponding applications. Coles (1986) advanced four possible explanations for anthelmintic failure among farm animals: (1) wrongfully under-dosing due to an underestimation of the animals' weight. Alternatively, a manufacturer's provided weight range of doses may cause animals at the higher end of the weight spectrum to be under-dosed. (2) Grazing animals in heavily contaminated pastures with GIN L_3, especially with highly pathogenic *H. contortus*, may portray general drug failure. (3) The presence of larval stages unaffected by drugs in use exacerbates infections. (4) Development of drug-resistant strains within GIN populations. The overall homozygous recessive alleles recorded herein portray serious levels, especially in the Highlands (RR = 24%). Consequently, farms in the Highlands hold significantly more Merino sheep per farmer than other ecological zones and also due to their predominant practice of transhumance. However, the Pearson Chi-square revealed no significant ($p > 0.05$) association between resistance-associated alleles and topographical divisions in Lesotho.

The results of the *H. contortus* sequences of the beta-tubulin isotype-1 partial gene in the present study have confirmed that the presence of benzimidazole resistance-carrying mutation is codon 200 (TAC) in Lesotho Merino sheep. From seven successfully sequenced isolates, there were two AR mutants at codon 200 of the beta-tubulin isotype-1 partial gene. The mutation at codon 200 (TTC to TAC), which is resistance-associated SNP was detected in other short sequences, which could not be used in the study. Alternatively, substitution mutations at codon 167 (TTC to TAC) and 198 (GAA to GCA) were not detected in the present study. This result corroborates records from studies that suggested mutation at codon 200 (TTC to TAC) was indeed the most widespread and prevalent (Onder *et al*., 2016).

The beta-tubulin isotype-1 gene also serves as a molecular marker for determining species identity because of the presence of conserved sites. Moreover, the molecular marker was

used in phylogenetic analysis, genetic diversity, population genetics, and in determining the occurrence, distribution, and intensity of AR-associated SNPs (Irum *et al.,* 2014; Emery, *et al.,* 2016; Mohanraj *et al.*, 2017). Furthermore, the molecular maker has also been functional in the study of parasite-host interactions, and gene flow. Two main methods of inferring phylogenetic analysis namely, distance-based (Neighbour-joining) and sequenced-based (Maximum likelihood) were used concurrently in the of beta-tubulin isotype-1 partial gene sequenced in the current study. The general structures of phylogenetic trees and the haplotype network analysis of the beta-tubulin isotype-1 partial gene revealed there was variation between the Lesotho isolates, notwithstanding their clustering together in Clade A. The marked variation was supported by relatively high pairwise values of evolutionary divergence recorded in beta-tubulin isotype-1 partial gene sequences from four ecological zones in Lesotho. It was not practical to deduce between mutant and wild-type genotypes from phylogenetic analysis or haplotype network analysis due to the overarching of several other SNPs among the sequences.

Haplotype diversity is an estimate of the probability that any two randomly chosen haplotypes in a population are different. The haplotype diversity of 1.000 ± 0.0760 in the beta-tubulin isotype-1 partial gene revealed total dissimilarity of genotypes among *H. contortus* isolate sequences across the study area. This therefore depicts major variability between the *H. contortus* isolates and suggests the possibility of low gene flow between ecological zones. Brasil *et al.* (2012) recorded haplotype diversity values ranging from 0.95 to 0.98 in Brazil, which were relatively lower than those recorded in the present study for *H. contortus*. Similarly, haplotype diversity values recorded in China by Shen *et al.* (2019) from wild blue sheep (0.952) and domestic sheep (0.926) were also lower than Brazil and the results of the present study. This resulted despite the haplotype diversity values still being regarded as relatively high. Blouin *et al.* (1995) suggested a generally high degree of genetic diversity among *H. contortus* populations that was symbolic of trichostrongylids. Consequently, high haplotype diversity is responsible for high genetic diversity in a population. The high haplotype diversity may in the context of *H. contortus* be directly related to its known high biotic potential, large population size, and short and rapid life cycle in *H. contortus* (Blouin *et al.,* 1995; Besier *et al.,* 2016). Nucleotide diversity (π), is another measure of genetic diversity that denotes an average pairwise difference among all sequence pairs of isolates in a population of interest. The seven Lesotho isolates had a total nucleotide

diversity of 0.01891, which was lower than values isolated from wild blue sheep (0.04172) and domestic sheep (0.03925) in China. Beech *et al.* (1994) also observed a remarkably high nucleotide diversity estimate of 0.091 for beta-tubulin isotype-1. Consequently, the low nucleotide diversity of the beta-tubulin isotype-1 partial gene in the present study suggests a relatively low level of gene flow among *H. contortus* subpopulations We only had seven sequences in our present work, which warrants a limited conclusion. Notwithstanding, the present determination exhibited the indicative gene flow mainly influenced by spatial distribution, environmental heterogeneity and the inherent biotic factors such as population size and short direct life cycle.

The Median-joining haplotype analysis was constructed from 26 beta-tubulin isotype-1 partial gene sequences of *H. contortus* from seven Lesotho and 19 GenBank sequences from around the globe. The analysis had 19 beta-tubulin isotype-1 haplotypes and one *T. circumcinta* haplotype. All seven sequences from Lesotho were assigned to distinct haplotypes. There were only three shared haplotypes in the Midian-joining haplotype analysis. This result supports the haplotype diversity value (1.000 ± 0.076), which implies that any two haplotypes chosen at random among the Lesotho isolates have a 100% likelihood that they are different genotypes. Beta-tubulin isotype-1 in the present study shows a relatively high diversity. Notwithstanding, Hap_7 (SRV_BT_24) and Hap_9 (LL_BT_13), the only two SNPs associated with benzimidazole-AR, were the closest among the Lesotho isolates. Similarly, the European haplotypes Hap_10, 11, and 12 from Turkey, Italy, the UK, and Switzerland, respectively, were closest in the analysis, which may suggest close sympatric status. Hap_6 from the Lesotho Highlands ecological zone exhibited uniqueness from the other six sequences on account of wide genetic distance by having the most mutations. This was further exemplified by phylogenetic tree analyses and that of the Median-joining haplotype analysis that showed relatively more genetic distance from Hap_6. Furthermore, the biggest estimate of evolutionary divergence value (0.03595) recorded in the present study was between the Hap_6 and BT_LL_12 from the Lowlands. Consequently, the population pairwise F_{ST} values were relatively higher between the Highlands and the Lowlands owing to the contribution by Hap_6 and probably also because of the low number of sequences used for the analysis.

Rearing a variety of animal species on a farm may also potentially influence gene flow among parasites through cross-infections resulting in relatively rapid parasite lifecycles, especially in animals grazed in common pastures. Moreover, the convergence of animals in the Highlands due to transhumances may also be a reason for the close genetic relationship among the Lesotho sequences, portrayed by clustering in the common clade. The negative Tajima's D in the present study suggested genetic diversity within the sequences was skewed toward rare genetic variants and could result in population expansion. It is therefore prudent to constantly monitor GIN populations for the presence and intensity of AR for an appropriate response and to develop tailor-made control methods. Parasite control initiatives should border on the integration of pasture management, chemical control, and diagnostic genotyping for beta-tubulin isotype-1 gene resistance owing to the interplay of several factors in a poor-resourced communal farming system. The robustness of molecular determination of AR, and its sensitivity to low levels of resistance makes it ideal for use in monitoring initiatives geared towards reduction and control of AR (Gallidis *et al*., 2012). Moreover, Ross *et al*. (1995) reported the protocols were applied to individual eggs, larvae, and adults and could still detect resistance as low as 1%. The use of DNA from larval coprocultures and the diagnosis of AR thereof provide evidence that benzimidazole resistance in *H. contortus* is a heritable trait. Consequentially, codon 200 of the beta-tubulin isotype-1 gene was invariably identified as the benzimidazole resistance-carrying mutant in Lesotho isolates. The Highlands registered the highest homozygous resistant alleles. Sequencing of the beta-tubulin isotype-1 partial gene also confirmed the presence of mutant genotypes.

The neutral theory of evolution affirms that the rate of genetic diversity in a population may reach equilibrium as determined by the balance between the input of a new type of variation and the loss of variation through the random genetic drift of alleles (Blouin *et al*., 1995; Salle' *et al*., 2019). Gene flow within and between populations is influenced by factors including the parasite life cycle, host migration, effective population sizes, and multiple host species in common grazing pastures (Blouin *et al*.,1995; Shen *et al*., 2019). Akkari *et al*. (2013) hypothesized a strong correlation between husbandry practices and the high genetic diversity in Tunisia. Moreover, Blouin *et al*. (1995) and Dey *et al*. (2019) reported that high gene flow within a parasite population influences the spread of resistant strains. Consequently, it became evident that where there was high refugia, frequent migration of

animals carrying more susceptible parasites into an area with high resistance may slow down the AR (Blouin *et al*., 1995). The highlands of Lesotho are characterized by relatively high livestock rates and transhumance practices, which consequently encourage the aggregation of host animals in the common pastures, both of which are a recipe for escalation of gene flow among their parasites. Hinney *et al*. (2020) recorded that sheep husbandry and climatic factors in the transhumant mountains in Austria increased drug selection for resistance by reducing refugia on pastures. This information can help us understand the factors that lead to the distribution and intensity of alleles responsible for AR and the factors that exacerbate the development of AR within the parasite population. F_{ST} of 11.475% in the study depicted the level of population differentiation between the Highlands and Lowlands of *H. contortus* between populations whereas nearly 88% was within populations. Moreover, the F_{ST} values of beta-tubulin Isotype-1 gene -(0.05556 and 0.11475) between sub-populations in the present study were comparable to those of Silvestre *et al* (2009) (0.3 to 0.183) from Southern France. The population structure was perceived to be a result of the high biotic potential of *H. contortus* and its adaptability in Southern France. Though Lesotho is a relatively smaller country, the local land and range management jurisdictions, especially in the Lowlands and some other parts of SRV and Foothills exhibit strict separation of animals, almost allopatric, existing in exclusively separated geographically hence diverse genetic differentiation and haplotype diversity of 1.000± 0.076. The genetic differentiation between sub-populations using beta-tubulin isotype-1 partial gene exhibited a low rate of gene flow between ecological zones and most explicitly between the Highlands and Lowlands. Consequentially, the beta-tubulin isotype-1 partial gene displayed an overall high diversity within populations and low differentiation between populations.

6.5. Conclusion

In conclusion, the results displayed the first evidence of molecular detection of anthelmintic resistance in Lesotho. The genotyping of benzimidazole-associated SNPs in codon 200 of the beta-tubulin isotype-1 gene yielded a positive AR diagnosis for *H. contortus* from naturally infected Merino sheep in four ecological zones of Lesotho. The null hypothesis that no benzimidazole resistance-associated alleles exist among *H. contortus* sub-populations from Merino sheep in Lesotho is therefore rejected. Consequently, the study revealed benzimidazole resistance-associated alleles were extensively distributed across

Lesotho's four ecological zones. The AS-PCR protocol successfully detected three different types of alleles of beta-tubulin isotype-1 responsible for benzimidazole resistance in *H. contortus* comprising homozygous susceptible (SS), heterozygous (SR), and homozygous resistant (RR). The result also exhibited genetic variations in benzimidazole resistance-associated SNP frequencies for different ecological zones of Lesotho. The results are indicative of the intensity of the resistance problem in Lesotho. It is envisaged that a broader initiative with a larger sample size may be considered in the future. The sequences from the present study were very diverse and resulted in different haplotypes, which may amount to single novel haplotypes.

CHAPTER 7

General discussion, conclusion, and recommendations

7.1. General Discussion

7.1.1. Introduction

Farmers were engaged from the beginning by outlining the aim of the project and requesting their consent for a questionnaire survey and for collecting faecal samples from sheep. This type of research initiative provides researchers with valuable information on agricultural activities performed in 'natural conditions' in comparison to controlled laboratory experiments. The approach was envisaged to promote better adoption and adaptability of the new technologies and subsequent recommendations for sustainable production and management among beneficiaries. Moreover, the initiative envisages improved healthy researcher and community engagement, where farmers also become researchers' partners and partake in socially acceptable, enhanced animal husbandry for sustainable and profitable livestock production. For this comprehensive study, multidisciplinary skills comprising expertise in genetics, bioinformatics, statistics, veterinary, clinical, and broad biological knowledge bases were sourced. In the present study, three types of GIPs identified by their eggs and oocysts from faecal samples included strongyles (64%), *Eimeria* spp. (18%), and *Monezia* spp. (1.3%). The dominance of GIN in Merino depicts the seriousness of and need for their research and subsequent management and control. The complexity of interaction between factors that affect the production and management of small ruminants warrants a thorough investigation of farming practices, drug use, AR development, range management, and animal movement. The present study provided an on-farm research reality of the epidemiological status *of H. contortus* across the four ecological zones of Lesotho.

7.1.2. Haemonchus contortus prevalence, distribution and abundance in Lesotho

Haemonchus contortus is the most pathogenic and economically important GIN of small ruminants worldwide (Almeida *et al*., 2018). The high strongyles' prevalence and infection intensity were reported for different farming areas in Lesotho (Moiloa *et al*., 2019; Mahlehla, 2021; Matsepe *et al*., 2021; Phalatsi *et al*., 2022). Understanding the epidemiological context

of *H. contortus* in Lesotho and its variation among ecological zones is of crucial importance for effective, region-specific parasite management and control. The insights gained may have far-reaching implications for a general understanding of the disease's etiology, veterinary interventions, farm management regimes, range management, and animal movement. Lesotho is a subtropical country and topographically subdivided into four major ecological zones, namely; the Highlands, Foothills, Lowlands, and the Senqu River Valley (SRV) (Mbata, 2001; Obioha, 2010). Lowlands are mainly warmer and accustomed to crop farming and human settlements, while the Foothills have a relatively balanced share of livestock and crop farming. Small ruminant-rearing communities are mostly concentrated in the Highlands (Obioha, 2010). The Highlands area is characteristically lower in temperature and exhibits relatively more livestock farming activities due to having vast rangelands. The seasons are different marked by cold and dry winters with warm and humid summers. The differences in climate are responsible for the seasonal dynamics of parasites such as *H. contortus*. Climate and change in season also play a vital role in vegetation which is a basis for nutrition in communally grazed livestock. The topographic differences are also used and managed to relieve the problem of overstocking by implementing rotational grazing regimes. Transhumance is one form of pasture rotation practice mainly in the Highlands, Foothills and SRV. *Haemonchus contortus*, a small ruminant parasite is distributed by this movement between pasture divisions and its prevalence is influenced by seasonality and vegetation. The stocking rates also affect the prevalence and intensity of parasites in the host and in the pastures.

The communal grazing system predominantly used in Lesotho is hypothesized to be the driving factor for the prevalence, distribution and intensity of *H. contortus*. The direct lifecycle of the parasite and the availability of some stages in the environment make *H. contortus* a perfect fit for a triad model of the parasite concept. The environmental stages on the pasture are the eggs to the infective stage. Animals are infected in the pastures and seasonal environmental conditions determine the length of the life cycle. Summer months therefore provide conducive conditions for shorter lifecycles. The Highlands had a high number of animals per farm and shared pastures. The Highlands as the major transhumance practicing area aggregate flocks in the mountain post during the high parasite activity in summer which increases the chances of pasture contamination and parasite infection. *H. contortus* is a widespread GIN of ruminants, with its epidemiology influenced by geographic

location, climate, high reproductive potential, and management practices (Emery *et al*., 2016; Besier *et al*., 2016). The high GIN prevalence (89%) and high average eggs per gram (1170.97) in the Highlands are most probably due to the multiplying effect of both stocking rates and high parasite activity timing. Moreover, the high mortality of sheep may be expected as infections increase. On the contrary, the Lowlands had the smallest number of sheep per farm, animals stayed in their grazing jurisdiction with more pasture divisions. The chances of infections may be expected to be low. Farms have diverse animal species and jointly use common pastures may contribute to elevated prevalence, distribution, and abundance of the GINs when not executed strategically. Moreover, high numbers of goats and cattle co-owned with Merino sheep in the present study area may constitute parasitic reservoirs which may perpetuate parasite existence.

7.1.3. Farmers' perspectives on gastrointestinal nematode management and control

The first section of the study dealt with farmer questionnaire surveys on their experience with farm management and control of GIN in their flocks. The study observed Merino sheep farming in Lesotho to be male-dominated (77.8%), and there were also more farmers (65%) with either non-informal or primary education. The questionnaire survey revealed that Merino sheep farmers in the Highlands had significantly more animals per farmer than in the rest of the ecological zones. Merino sheep farming in Lesotho is mainly of extensive and predominantly used communal pastures while intensive farms comprise about 10%. Extensive farming is characterised by low-input farming in which animals are mainly grazed in communal pastures with sporadic food supplementation. Small ruminant farms in Lesotho comprise a variety of animal species with Merino sheep a dominating species. The number of Merino sheep correlated substantially with that of goats depicting farms with mixed animal species a common phenomenon in Lesotho. There was a remarkable disparity regarding animal movement and range management, in which the Highlands, SRV, and Foothills predominantly exhibited transhumance, except for the Lowlands.

7.1.4. Characterisation and population genetics of Haemonchus contortus in Lesotho

The diagnostic protocol only catered to the parasites that could be identified using the flotation principle and McMaster slide. *Haemonchus contortus* was discovered to be an overwhelmingly widespread GIN in Lesotho. Both the prevalence and abundance of GIN were found to be higher and statistically significant for the Highlands than in all other

ecological zones. Notwithstanding the probability of finding other GIN species in Merino sheep, the recent results strongly exhibit the overwhelming dominance of *H. contortus* in Lesotho. The faecal matter was further processed for coproculture to identify the L_3 at the species level. L_3 isolates from coprocultures revealed 100% *H. contortus* when identified morphometrically. All collection sites in the present study were positive for *H. contortus*. The present findings confirm that *H. contortus* was the major GIN and was distributed across all ecological zones. All L_3 identified morphometrically were subjected to molecular characterization using ITS-2 of the ribosomal DNA, and conclusively identified as *H. contortus*. The homology of Lesotho isolates, when compared with GenBank sequences, was between 96 and 100% for *H. contortus*.

The diversity and population dynamics of *H. contortus* were investigated in the four ecological zones of Lesotho. The morphometrical L_3 identification results were in congruence with those of the molecular protocol. The phylogenetic and haplotype analyses revealed shared haplotypes by Lesotho isolates across the four ecological zones. 20 sequences in the current study yielded four haplotypes. There were no specific topographic patterns of haplotype distribution in the study. Furthermore, shared haplotypes with other world sequences also showed very weak continental patterns using the IST-2 molecular marker. The result demonstrates a relatively high probability of gene flow among isolates in the Highlands due to transhumant animal aggregation as portrayed by ITS-2. There was a low likelihood of gene flow based on separate village pasture administration and the inexistence of transhumance in the Lowlands. Moreover, the Highlands also had significantly more animals per farmer than all ecological zones. The hypothesis was further supported by the relatively high haplotype diversity recorded for the Lowlands ecological zone.

7.1.5. Intensity and distribution of benzimidazole resistance alleles in Haemonchus contortus in Lesotho

Historically, benzimidazole has been the most common drug group used for treating GIN in ruminants (Gilleard and Beech; Gilleard, 2006). Intensive use of anthelmintic drugs was reported among small ruminant farms in Lesotho, of which the benzimidazole group predominates (Moiloa *et a*l., 2020; Mahlehla *et al*., 2021). *Haemonchus contortus* is regarded as a model GIN for AR research and population genetics due to the availability of molecular-based data and well-defined anthelmintic-resistant molecular markers, a good basis for its future control in Lesotho. Benzimidazole drugs were the most used, and farmers generally increased dosing frequency over five years. There is evidence that the frequency of drugs has increased and that over the same period, the use of the benzimidazole group seemingly dropped and gave way to levamisole and ivermectin. This showed that irrespective of the low literacy of farmers in Lesotho, they could still change drug brands when they observed recurring symptoms of helminthiasis.

There were four main drug types used against GIN by farmers in Lesotho, namely benzimidazole, Levamisole, Ivermectin, and Nuclosamide. Benzimidazole was the main drug of choice for Lesotho farmers. This supported the need to investigate the intensity and frequency at which farmers administer drugs on the farms, as there is a suspicion of multidrug resistance development in Lesotho. Apart from the anthelmintic drugs, over half of the respondents administered ethnoveterinary and indigenous treatments against GINs and other common ailments. Herbal and Indigenous treatments are common among resource-poor rural farming communities. The knowledge has been passed from one generation to another (Ndlela *et al*., 2022).

The study revealed the presence of alleles responsible for the development of benzimidazole resistance in GINs in codon 200 of the beta-tubulin isotype-1 gene of *H. contortus*. The beta-tubulin isotype-1 gene was then sequenced to establish the existence of the resistance point mutations. The evaluation of *H. contortus* diversity was also investigated using the beta-tubulin isotype-1 partial gene. The present initiative presented the first findings on the occurrence, prevalence, and distribution of benzimidazole-associated SNPs responsible for

AR development in Lesotho. Molecular detection of AR by AS-PCR has proved to be a faster, more cost-effective, and more reproducible protocol than the *in vivo* and *in vitro* methods. Of the 89 L_3 sample isolates from four ecological zones, there were overall 51.7% homozygous susceptible (SS), 32.6% heterozygous (SR), and 15.7% homozygous resistance (RR) alleles. The results suggest the presence of heterozygosity, a concept in population genetics and evolutionary biology is vital in genetic diversity, which is essential for the adaptability and survival of populations in constantly changing environments. The above alleles were present in all four ecological zones, though at varying frequencies, with the Highlands registering a relatively higher frequency of resistant strains (24%), while the Lowlands (9%) were the lowest. Notwithstanding the relatively small sample size in our study, the disparity in resistance among ecological zones resulted from the diverse range management and animal movement regimes in the ecological zones.

The high homozygous susceptible alleles (84.3%) in the current study may suggest a high possibility of parasites in refugia. Refugia, if investigated intensely may be used strategically as a dilution factor against the spread of resistant strains. Consequently, homozygous susceptible alleles can reduce the rate of progression and spread of resistance-associated SNPs within parasite populations. This therefore suggests that any gene recombination with homozygous susceptible (SS) strains will result in phenotypically susceptible strains to the drug in question. The rangelands in the Highlands are open for animals from other ecological zones, perpetuating gene flow and consequently the spread of resistant alleles among parasite populations. The anthropogenic influenced migration in search of pastures and livestock trading are major contributors to the large effective population and hence the current *H. contortus* population structure in Lesotho.

Merino sheep farmers generally attained low education exposure; a situation presumed to be associated with risk against adherence to optimum production standards. Training on the nature and usage of anthelmintic drugs needs special attention to avert the consequences of haphazard administration. The development of baseline information and regular monitoring of AR in communal farm areas are essential for sustainable parasite control. This will assist the veterinarians, Livestock Extension officials, and land and range management personnel to develop effective communication and tailor-make strategies for effective control and

management of GIN. Consequently, the unique character of the Highlands may need special attention in dealing with parasites and AR dynamism.

Anthelmintic resistance development against GINs has been reported as an inevitable outcome in farms that consistently use anthelmintic drugs against GINs worldwide (Gilleard *et al*., 2006). Mphahlele *et al*. (2021) observed that AR development among the parasite populations resulted from extensive and haphazard drug administration insufficient knowledge and a lack of proper guidance on anthelmintic drug administration by the poor-resourced farmers in Limpopo, South Africa. Consequently, Jabbar *et al*. (2006) also reported that an increase in AR among GINs in ruminants was an indication of failed anthelminthic control programs.

7.2. Limitations of the Study

The study was a cross-sectional type in which samples were collected one time. A questionnaire survey was face-to-face with telephonic interviews for farmers we missed during visits to their villages. This has an inherent bias as we could have made observations to confirm our conversation with the respondents in face-to-face interviews. We had different numbers of respondents where the lowlands had more and SRV had the least, the disparities may have implications on statistics and the overall research analyses. The difference also has some inherent bearing on results and analysis. Sheep dentition and owner memory were used to estimate sheep age. Due to inherent institutional capacity, our samples and reagents were always at risk of compromised cold chain due to prolonged power outages, as there was no backup generator. Sending DNA samples to South Africa and Europe posed a challenge of long freight which eventually compromised the integrity of our samples sequencing results. Our molecular work had a relatively low number of samples.

7.3. General Conclusion

Haemonchus contortus was the most abundant and widely distributed GIN in Merino sheep in Lesotho with the Highlands having significantly higher intensity than other ecological zones. There was no significant difference between burdens of GIPs by Merino sheep age groups. Transhumance was most predominant among the Highlands farmers followed by Foothills and SRV and was uncommon in the Lowlands. There was an overwhelming use of

anthelmintic drugs among respondents in the present study. The benzimidazole resistance-conveying alleles were detected in all ecological zones in Lesotho by AS-PCR. The Highlands had relatively higher resistance alleles associated with gene point mutations responsible for AR. The findings in the present study are important for the general conceptualization of *H. contortus* epidemiology in Merino sheep in Lesotho. The study revealed that *H. contortus* possesses varying benzimidazole resistance-associated SNPs in codon 200 of the beta-tubulin isotype-1 gene in Lesotho. All alleles (SS, SR, and RR) were present in *H. contortus* infecting Merino sheep across four ecological zones at different intensities. In the present study, a relatively high occurrence of heterozygous alleles (SR) was found across Lesotho. The heterozygous alleles have the potential to intensify the levels of AR development. The intensity of benzimidazole is considered exceptionally high and may need special attention to manage and control. Highlands possessed significantly more animals per farmer and were also predominant in practicing transhumance, which aggregates animals in the mountain animal posts for several months during summer periods. The transhumance system results in the seasonal sympatry of host animals in the mountain animal posts during the summer grazing, especially when parasites are at their peak activity. The genetic diversity of the beta-tubulin isotype-1 gene of *H. contortus* is important to critically understand the dynamics of benzimidazole resistance intrapopulation gene flow within Lesotho. The user-friendliness of molecular protocols is ideal for Lesotho due to their ease of application especially for the mountainous hard-to-reach places where animals converge during summer grazing. Conventional methods are labour-intensive and may require multiple visits for faecal sample collections to complete the full regime for the protocol. The epidemiological status of *H. contortus* has shown that its dynamism was dependent on the occurrence and burdens of parasites, movement and stocking rates of the Merino sheep, and the environmental conditions displayed through different topographical stratus in our study area. There was also the influence of anthropogenic factors in terms of management regimes across the four ecological zones in Lesotho.

7.4. Recommendations and Future Perspectives

- Several methods of drug efficacy tests such as *in vivo, in vitro* and molecular anthelmintic resistance protocol are recommended to be carried out concurrently

across the country. Real-time PCR and RFLP assays may also be used in the detection and monitoring of SNPs responsible for AR in *H. contortus*.

- Due to the high likelihood of the occurrence of resistance-associated alleles among *H. contortus* populations, stringent animal movement regulations need special attention. This may assist in the strategic prevention and control of AR spread among Lesotho ruminants.
- Lesotho is dominated by farms that raise mixed animal species and co-grazed in common communal rangelands. Farm animals such as goats, cattle, donkeys, and horses were found in substantial numbers hence a need to be included in regular GIN screening and AR development monitoring for prompt control.
- Harmonisation of farm management practices such as drug administration, timing of transhumance, stocking rates, and regular AR checks are recommended to avert overgrazing and improve parasite prevention and control programs.
- There is a need to investigate the in-refugia GINs on prevalence, intensity, seasonal abundance and presence of anthelmintic resistance from the communal rangelands for comprehensive knowledge and mounting appropriate management and control tools.

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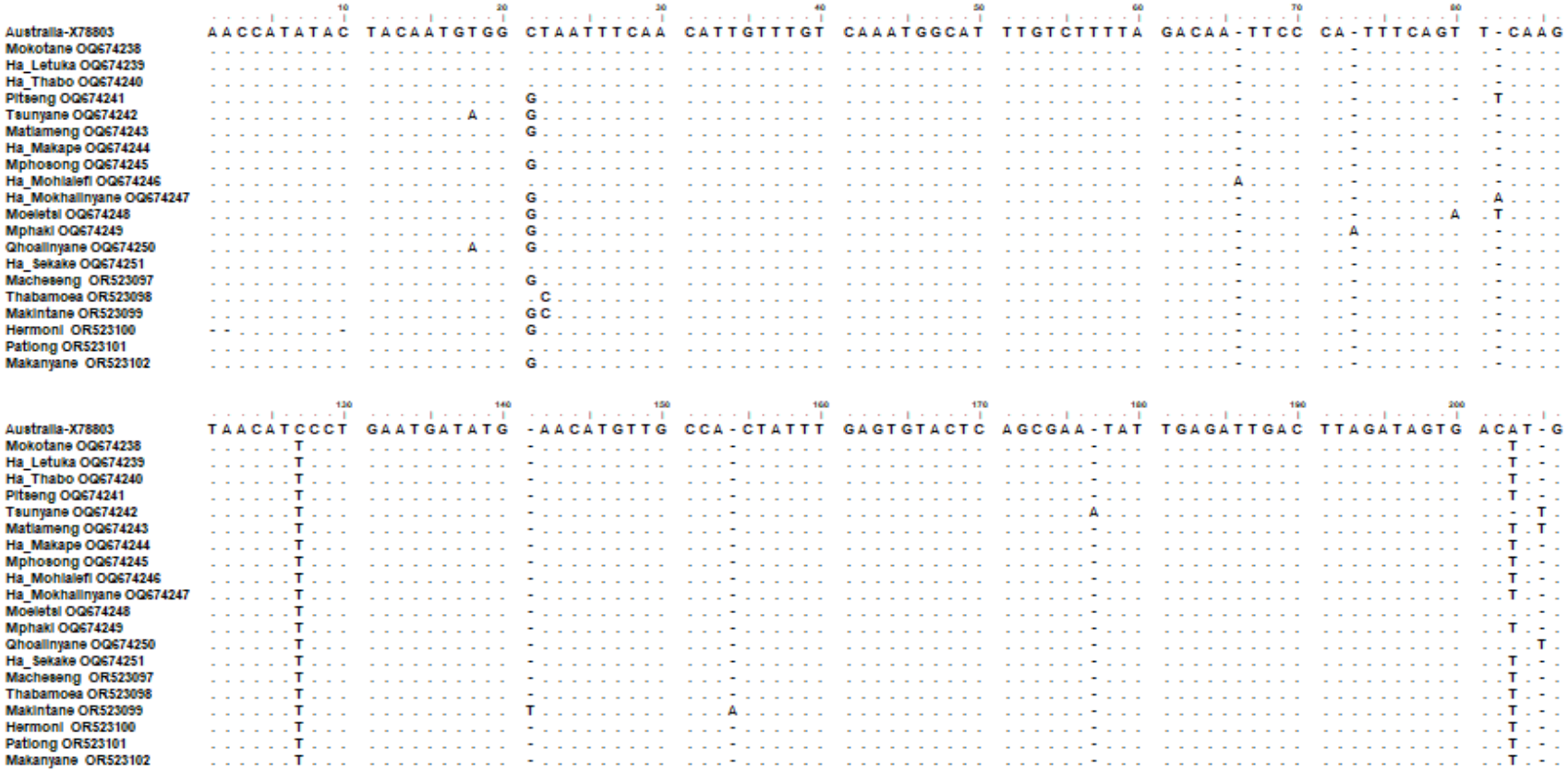
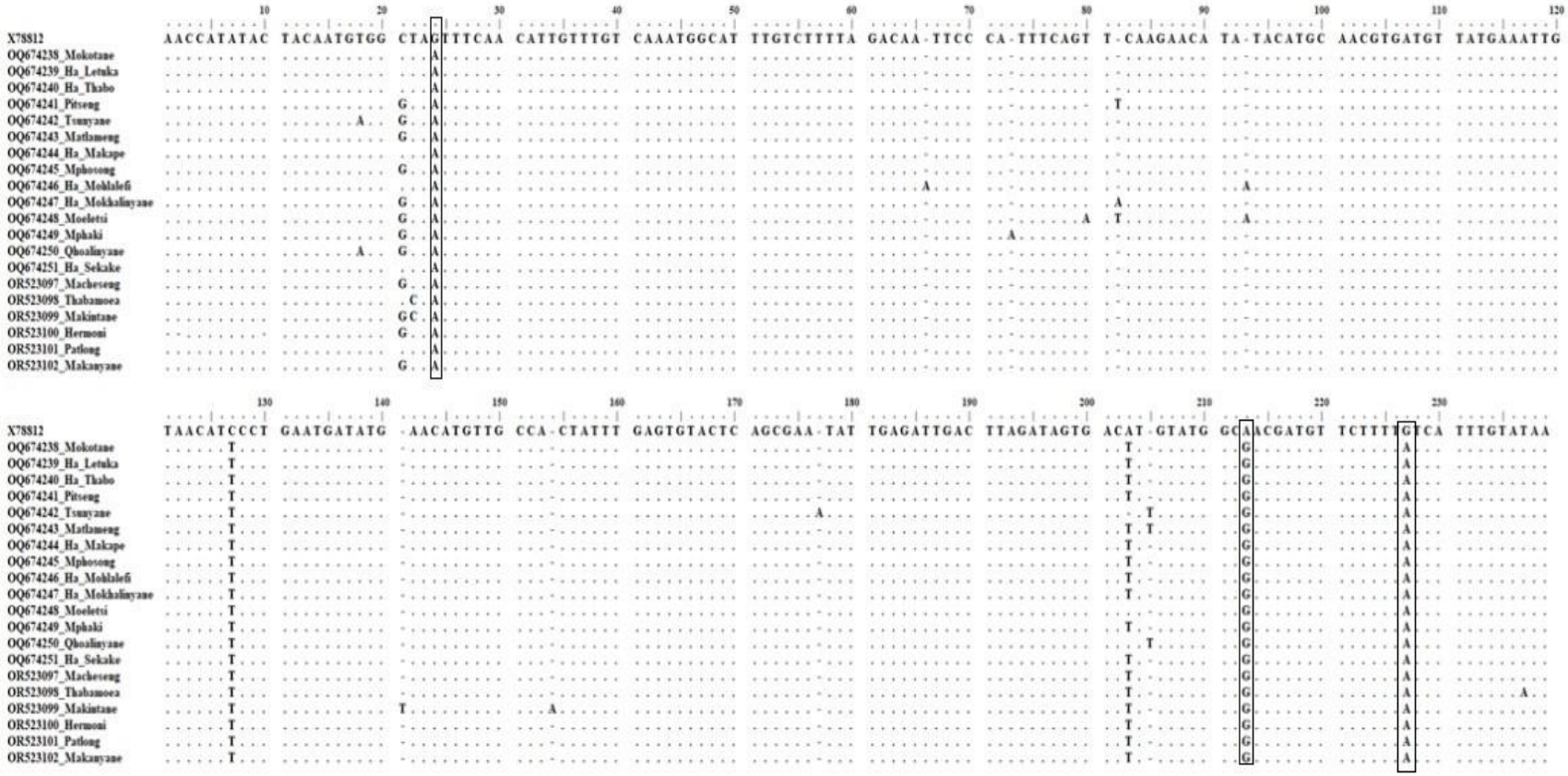
APPENDICES

Appendices A: Nucleotide sequence polymorphism of 231 bp of a complete segment of ITS-2 of the ribosomal DNA *Haemonchus contortus* showing nucleotide variations between Lesotho isolates using *H. contortus* accession number X78003



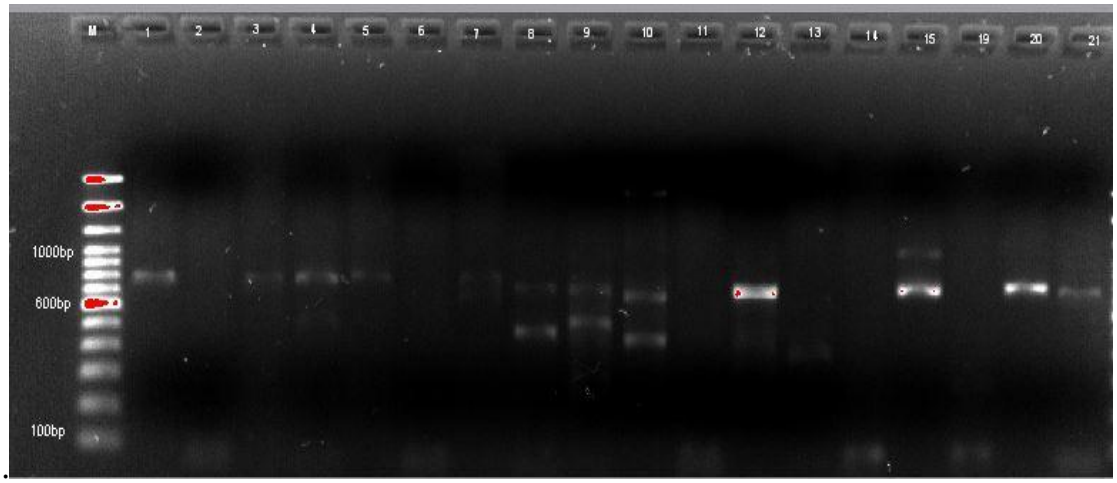
Appendix B: Multiple alignment of complete *Haemonchus contortus* ITS-2 gene of Lesotho isolate sequences using *H. placei* (accession number X78812) as reference sequences. The graphical image depicts three main distinct differences between *H. contortus* and *H. placei* SNPs (24 (G), 203 (A), and 217 (G),).



Appendix C: The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Tamura 3-parameter model [1]. This analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 624 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

	X80046	BT_HL_01	BT_HL_04	BT_FH_10	BT_LL_12	BT_LL_13	BT_SRV_20	BT_SRV_24	FJ981631	DQ469247	DQ469250	AY749129
X80046												
BT_HL_01	0.03619											
BT_HL_04	0.03955	0.02115										
BT_FH_10	0.03288	0.00806	0.02942									
BT_LL_12	0.04132	0.02445	0.03612	0.02610								
BT_LL_13	0.04812	0.01785	0.02113	0.02610	0.02279							
BT_SRV_20	0.04301	0.00644	0.01621	0.01456	0.02777	0.01130						
BT_SRV_24	0.04642	0.01622	0.01948	0.02444	0.02445	0.00160	0.00968					
FJ981631	0.04639	0.01621	0.01949	0.02444	0.02114	0.00806	0.00968	0.00644				
DQ469247	0.04812	0.01785	0.02113	0.02610	0.02279	0.00968	0.01130	0.00806	0.00161			
DQ469250	0.04812	0.01785	0.02113	0.02609	0.02279	0.00968	0.01130	0.00806	0.00161	0.00322		
AY749129	0.03288	0.01784	0.03109	0.01457	0.02610	0.02279	0.02445	0.02114	0.02444	0.02610	0.02610	
KX246648	0.01785	0.03800	0.02950	0.04304	0.04308	0.03625	0.03122	0.03459	0.03455	0.03625	0.03625	0.04477
KX246656	0.03288	0.01784	0.03109	0.01457	0.02610	0.02279	0.02445	0.02114	0.02444	0.02610	0.02610	0.00000

Appendix D: The image of agarose gel electrophoresis of *H. contortus* larvae amplicons showing beta-tubulin isotype-1 partial gene PCR product of ±750 bp. 1.5% agarose gel was run on 100 volts for 50 minutes. DNA ladder of 100 bp was used



espi

Appendix E: Beta-tubulin isotype-1 partial gene sequences based on 627 bp from the GenBank used in the phylogenetic analysis and haplotype network analysis with Lesotho isolates

	GENBANK ACCESSION NUMBER	TYPE OF STRAIN	COUNTRY OF ORIGIN
1	FJ981632		SWITZERLAND
2	FJ981631		SWITZERLAND
3	DQ469247		AUSTRALIA
4	DQ469250		AUSTRALIA
5	AY749129		AUSTRALIA
6	KX246648		USA
7	KX246656		USA
8	KX246666		USA
9	OP868806		ITALY
10	OP868805		ITALY
11	KJ410523		TURKEY
12	KJ410522		TURKEY
13	OP642037		POLAND
14	JF784609		MEXICO
15	LS997562		-
16	X80046		-
17	X67489		-
18	KF683619	(*T. circumcinta*)	Outgroup

Appendix F: Alignment of BT_ HL_01 (susceptible) with isolate accession no. DQ469247 (susceptible strain) shows 98% homology.

Haemonchus contortus clone H2 beta-tubulin gene, partial sequence

Sequence ID: DQ469247.1 Length: 811 Number of Matches: 1

Range 1: 53 to 679 GenBank Graphics Next Match

Score	Expect	Identities	Gaps	Strand
1086 bits(588)	0.0	614/627(98%)	0/627(0%)	Plus/Plus

```
Query  1    GATAATTACGTGTTTGGCCAGTCAGGAGCGGGTAACAATTGGGCGAAGGGCCACTATACT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  53   GATAATTACGTGTTTGGCCAGTCAGGAGCGGGTAACAATTGGGCGAAGGGCCACTATACT  112

Query  61   GAGGGAGCCGAGCTAGTTGATAACGTCATAGACGTTGTACGCAAAGAAGCTGAAGGTTGT  120
            |||||||||||||||||||||||| |  ||||||||||||||||||||||||||||||||
Sbjct  113  GAGGGAGCCGAGCTAGTTGATAACATACTAGACGTTGTACGCAAAGAAGCTGAAGGTTGT  172

Query  121  GATTGCCTTCAGGTACTGCGTTCATCAACAATTTACAGCTTCAACTCTTATGAGTGAATA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  173  GATTGCCTTCAGGTACTGCGTTCATCAACAATTTACAGCTTCAACTCTTATGAGTGAATA  232

Query  181  AATTTCAATTTGTACTCAGGGCTTCCAATTGACGCATTCACTTGGAGGAGGCACTGGATC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  233  AATTTCAATTTGTACTCAGGGCTTCCAATTGACGCATTCACTTGGAGGAGGCACTGGATC  292

Query  241  TGGAATGGGCACTTTGTTAATTTCAAAAATTCGTGAAGAGTACCCTGATAGAATTATGGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  293  TGGAATGGGCACTTTGTTAATTTCAAAAATTCGTGAAGAGTACCCTGATAGAATTATGGC  352

Query  301  TTCGTTCTCCGTTGTTCCATCACCCAAGGTGAGATCGTGTTAATCTTTCCTCTTTTCCTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  353  TTCGTTCTCCGTTGTTCCATCACCCAAGGTGAGATCGTGTTAATCTTTCCTCTTTTCCTA  412

Query  361  AATTGTGTATTTGAATTATTTATCCTCATGAAAAATTCAAGTAGAAATAAGTCTCACCAT  420
            |||||||||||||||||||||||||||||| ||||||||||||    ||||||||||||||
Sbjct  413  AATTGTGTATTTGAATTATTTATCCTCATCAAAAATTCAAGTCTTAATAAGTCTCACCAC  472

Query  421  CTGTAAACATGTGAAAGGAAGATGTTTTAAGGTATCCGACACTGTTGTGGAACCCTACAA  480
            |||||||| || |||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  473  CTGTAAACGTGCGAAAGGAAGATGTTTTTAGGTATCCGACACTGTTGTGGAACCCTACAA  532

Query  481  TGCTACCCTTTCCGTCCATCAATTGGTAGAAAACACCGATGAAACATTCTGTATTGACAA  540
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  533  TGCTACCCTTTCCGTCCATCAATTGGTAGAAAACACCGATGCAACATTCTGTATTGACAA  592

Query  541  CGAAGCTCTGTATGATATCTGCTTCCGCACTTTGAAACTCACAAATCCAACCTATGGAGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  593  CGAAGCTCTGTATGATATCTGCTTCCGCACTTTGAAACTCACAAATCCAACCTATGGAGA  652

Query  601  TCTCAACCACCTTGGTAAATGTTGTTA  627
            |||||||||||||||||| ||||||||
Sbjct  653  TCTCAACCACCTTGGTAATTGTTGTTA  679
```

Appendix G: BT_HL_04 (susceptible strain) with isolate accession no. X67489 (resistant strain) shows 96% homology.

Haemonchus contortus GRU-1 gene for beta-tubulin

Sequence ID: X67489.1 Length: 4531 Number of Matches: 1

Range 1: 2184 to 2809 GenBank Graphics ▼ Next Match ▲

Score	Expect	Identities	Gaps	Strand
1007 bits(545)	0.0	600/627(96%)	1/627(0%)	Plus/Plus

```
Query  1     GATAATTACGTGTTTGGCCAGTCAGGAGCGGGTAACAATTGGGCGAAGGGCCACTATACT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2184  GATAATTACGTGTTTGGCCAGTCAGGAGCGGGTAACAATTGGGCGAAGGGCCACTATACT  2243

Query  61    GAGGGAGCCGAGCTAGTTGATAACGTCATAGACGTTGTACGCAAAGAAGCTGAAGGTTGT  120
             ||||||||||||||||||||||||||  |||||||||| ||||||||||||||||||||||
Sbjct  2244  GAGGGAGCCGAGCTAGTTGATAACGTATTAGACGTTGTCCGCAAAGAAGCTGAAGGTTGT  2303

Query  121   GATTGCCTTCAGGTACTGCGTTCATCAACAATTTACAGCTTCAACTCTTATGAGTGAATA  180
             ||||||||||||||||||  |||||||||||||||||||||||| | ||| |||||||
Sbjct  2304  GATTGCCTTCAGGTACTGACTTCATCAACAATTTACAGCTTCAACTTTGATGTGTGAATA  2363

Query  181   AATTTCAATTTGTACTCAGGGCTTCCAATTGACGCATTCACTTGGAGGAGGCACTGGATC  240
              |||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2364  CATTTCAATTCGTGCTCAGGGCTTCCAATTGACGCATTCACTTGGAGGAGGCACTGGATC  2423

Query  241   TGGAATGGGCACTTTGTTAATTTCAAAAATTCGTGAAGAGTACCCTGATAGAATTATGGC  300
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2424  TGGAATGGGCACTTTGTAAATTTCAAAAATTCGTGAAGAGTACCCTGATAGAATTATGGC  2483

Query  301   TTCGTTCTCCGTTGTTCCATCACCCAAGGTGAGATCGTGTTAATCTTTCCTCTTTTCCTA  360
             |||||||||||||||||||||||||||||||||||||||||||||| || ||||||||
Sbjct  2484  TTCGTTCTCCGTTGTTCCATCACCCAAGGTGAGATCGTGTTAATCTTTGCTTTTTTCCTA  2543

Query  361   AATTGTGTATTTGAATTATTTATCCTCATGAAAAATTCAAGTAGAAATAAGTCTCACCAT  420
             |||||||||||||||||| ||||||||||||  || ||||| ||||||||||||||||
Sbjct  2544  AATTGTGTATTTGAATTACTTATCCTCATGAAG-ATCCAAGTTGAAATAAGTCTCACCAC  2602

Query  421   CTGTAAACATGTGAAAGGAAGATGTTTTAAGGTATCCGACACTGTTGTGGAACCCTACAA  480
             |||||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  2603  CTGTAAACATGTGAAAGGAAGATGTTTTAAGGTATCCGACACTGTCGTAGAACCCTACAA  2662

Query  481   TGCTACCCTTTCCGTCCATCAATTGGTAGAAAACACCGATGAAACATTCTGTATTGACAA  540
             |||||||||||||||||||||| ||||||| ||||||||||||||| ||||||||||||
Sbjct  2663  TGCTACCCTTTCCGTCCATCAACTGGTAGAGAACACCGATGAAACATACTGTATTGACAA  2722

Query  541   CGAAGCTCTGTATGATATCTGCTTCCGCACTTTGAAACTCACAAATCCAACCTATGGAGA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2723  CGAAGCTCTGTATGATATCTGCTTCCGCACTTTGAAACTCACAAATCCAACCTATGGAGA  2782

Query  601   TCTCAACCACCTTGGTAAATGTTGTTA  627
             |||||||||||||||||| |||| |||
Sbjct  2783  TCTCAACCACCTTGGTAATTGTTATTA  2809
```

Addendum H: Multiple sequence alignment of *H. contortus* beta-tubulin isotype-1 (626 bp) from Lesotho using accession number susceptible variant sequence X80046 as a reference. The segment corresponds to the partial gene starting from part of exon 3 to exon 5 with associated intervening introns.

Appendix I: A questionnaire used in the study of the perceptions of farmers on Merino sheep management, GINs control and development of anthelmintic resistance in Lesotho. A PhD study in the Department of Animal Science, Faculty of Agriculture in National Universiy of Lesotho

Questionnaire Number	
Name of interviewer/ Lebitso la ea botsang	
Date of interview	
Name and Surname: Lebitso le fane	
Physical Address: Sebaka sa bolulo	
Cell phone number:	
Village; Motse	
Local community council	
Constituency:	
District/Setereke	
Locality Co-ordinates	

1. Gender and age group

	Gender	Male Bonna			Female Bot'seheli		
	Age group Lilemo tsa sehoai	18- 25	26- 35	36- 45	46- 55	56- 65	66+

2. What is your level of education? O kene sekolo ho fihla kae.

Highest education Level reached	None/no formal education	
	Primary	
	Secondary	
	High school	
	Tertiary	
	Other (specify)	

3. How long have you been rearing sheep? O na le nako e kae o ruile linku?

Less than 5 years Ka tlasa lilemo tse hlano (5)		From 6 to10 years Lilemo tse 5 ho idsa ho tse 10		More than 10 years Ka holimo ho lilemo tse leshome	

4. Which other animal species do you keep, and how many are they? Ke mefuta efe ea liphoofolo eo o e ruileng le lipalo tsa tsona.

Species/Mofuta oa phoolofolo	Number of animals/Palo ea liphoofolo
Cattle/likhomo	
Donkeys/litonki/	
Horses/Lipere	
Goats/lipoli	
Sheep/linku	
Pigs/likolobe	
Tse ling.	
Total	

5. What feeding regime do you practice? U sebelisang ho fepa liphoofolo?

Feeding	Select Appropriate Options
Zero grazing (100% feeding)	
Communal grazing / lekhulong	
Secluded grazing (not shared)/ polasing	
Supplements /phepo tlatsetso	

Grazing and supplements/ lekhulo le phepo tlatsetso	
Also use transhumance (Meraka)	
Other (Explain)	

6. If transhumance is used at which months? Liphoofolo li isoa nakong efe meraka

Month	J	F	M	A	M	J	J	A	S	O	N	D
Tick												

7. Are range management enforcers strict. Na taolo ea makhulo e tsepame taolong?

Yes/Ee	
No/Chee	

8. Do farmer adhere to management schedule? Na lihoai li ikobela taolo ea makhulo?

Yes/Ee	
No/Chee	

9. Liphoofolo tse eang lekhulong la heno li kaba kae?

Number	≤100	Between 500 and 1000	≥1000	Other/ho hong, Hlalosa
Tick				

10. Are you aware of internal worms that infect livestock? Na o tseba ka manyooa a ts'oaetsang liphoofolo?

Yes/Ee	
No/Chee	

11. Do you know how animals are infected with gastrointestinal nematodes? Na o tseba kamoo liphofolo li ts'oaetsoang ke monyooa ka teng?

Yes/Ee	
No/ Chee	

12. If the answer is yes in the above question, explain how animals become infected with gastrointestinal nematodes! Ha karabo e le ee ka holimo, Hlalosa!

How animals get infection	Tick
Parasites co-exist with hosts from birth	
From water they drink	
From other animals	
From pastures	
Do not know	

Other (Explain)	

13. Can you identify an animal infected with worms? Na o tseba ho hlalohanya/ho bona ha phoofolo e ts'oeroe je manyooa?

Yes/Ee	
No/ Chee	

14. If the answer for the above questions is yes: mention symptoms that you know on GIN infection. Ha karabo e le ee kaholimo hlalosa mats'oao ao o a tsebang a liphoofolo (linku) tse ts'oeroeng ke manyooa.

a)	
b)	
c)	
d)	
e)	
f)	

15. How many animals showed symptoms of helminthiasis in the last 6 months? Ke liphoofolo tse kae tse bontsang matsoao ha ho tsoaroa ke manyooa khoeling tse tseletseng tse fetileng.

Lambs Makonyana	
Adult Sheep/ Tse kholo	

16. Are you able to identify an animal that died due of gastrointestinal nematodes? Na o ka khona ho hlalohanya le ho tseba phoolofolo e bolailoeng ke manyooa?

Yes/Ee	
No/ Chee	

17. If yes, what are signs of a sheep which died of gastrointestinal nematodes? Mats'oao a liphoofolo (linku) tse bolauoeng ke manyooa.

a)	
b)	
c)	
d)	

18. How many animals died from gastrointestinal nematodes in your flock in the last year? Ke liphoofolo tse kae tse bolailoeng ke manyooa?

Lambs/Makonyana	
Adult/Tse kholo	

19. Which months of the year do the animals usually show the signs of worm infection? Ke likhoeli life tseo liphoofolo li ts'oaroang ke manyooa?

Month	J	F	M	A	M	J	J	A	S	O	N	D

Tick												

20. Which months of the year do administer anthelmintic drugs on animals? Ke likhoeli life tseo liphoofolo li fuoang moriana oa manyooa?

Month	J	F	M	A	M	J	J	A	S	O	N	D
Tick												

21. Do you treat your animals with anthelmintic drugs? A o phekola liphoofolo tsa hao meriana ea manyooa ea sekhooa?

Yes/Ee	
No/ Chee	

Where do you buy/get anthelmintic drugs for your animals? O fumana meriana ee ea sekhooaq kae?

Drug outlets		Animal extention officers		Farmers association		Other/ho hong, Hlalosa	

22. Do you ever change the drug brand that you use? Na o ee o fetole moriana oo o li fang liphoofolo tsa hao khahlanong le manyooa??

Yes/Ee	
No/Chee	

23. If yes how often do you change a drug? Ha karabo e le ee, o e fetola ha kae? Hobaneng o fetola?

Never changed	
Once in two years	
Once a year	
Once a season	
Other (explain)	

24. Do you know how much medicine (dosage) to give to your animals? A o tseba tekano/semetho sa meriana ee ka moo lokelang ho e fa liphoofolo?

Yes/Ee	
No/Chee	

Can you read the drug instructions by yourself? Na o khona ho ipalla tataiso ea tsebeliso ea moriana

Yes/Ee	
No/Chee	

25. If no, how do you know the amount of drug to administer on your animals? Ha e be Karabo chee! o etsa eng ho fana ka sekepele sa moriana liphoolofolong tsa hao.

Method of determining	Tick appropriate
Get assistance from extension officers/ Ke thusoa ke basupisi/lingaka	
Get assistance from fellow farmers/Ke thusoa ke barui ba bang	
Give rough estimation of amount/Ke fana ka khakanyo eo ke e nahanang	
Ask people to do it for me.	
Do not know	
Other (Explain)	

26. Do you weigh your animals before dosing? Na o nka boima ba Liphoofolo tsa hao pele o li fa moriana/litlhare?

Yes/Ee	
No/Chee	

27. Do you know if the remedies are working/effective? Na o ee o bone litlhare/meriana eo e folisa?

Yes/Ee	
No/ Chee	

28. What result did you see after drug administration on your animals? Ke eng eo o e bonang liphoofolong kamora ho li fa moriana?

Result after drug administration	Tick
Animals get better/liphoofolo lia fola	
No change/ha ho phetoho	
Other/explain	

29. List anthelmintic drugs you have been using in the last five years? Bolela meriana ea manyooa eo o sa leng o e sebelisa liphoofolong lilemong tse hlano tsa ho feta?

a.	
b.	
c.	
d.	
e.	
f.	
g.	

30. Which of the above anthelmintic drugs did you use most recently? Ke ofe moriana oo sebelisitseng khetlo ha o qetela?

31. Where have you been getting advice for anthelmintic use. O fumana botataisi baa meriana le ts'ebeliso ea oona kae?

a.	Radio programmes/sea-le-moeeng	
b.	Peer farmers (barui ba bang)	
c.	Animal extension services/bosupising	
d.	Training workshop/thupelong	
e.	Own initiatives/ke ipatletse leseli	
f.	Mokhatlong oa barui/association	
g.	Other (specify)	

32. O reka meriana ea manyooa a bo ka selemo?

Amount	≤M500	Between M500 and M1000	≥M1000	Other/ho hong, Hlalosa
Tick				

33. How do you store anthelmintic drugs? O boloka meriana ea liphoofolo tsa hao joang?

Drug use and Storage type	Buy only required amount/Ke reka o lekaneng hore o fele	in my house/ Ka tlung	Sebakeng se khethehileng sa meriana/ place conducive for anthelmintics	Other explain/ho hong, hlalosa
Tick				

34. How often did you administer the medicine to your livestock last year? O phekola sebelisitse moriana ha kae selemong se tsoa feta liphoofolo tsa hao khahlanong le manyoa.

Frequency	Once a year/ Hang selemong	Twice a year/ Habeli ka selemo	Thrice a year/ Ha raro ka selemo	Other/makhetlo a mang hlalosa
Tick				

35. How often did you administer the drugs on your livestock five years ago? If other, please specify. O phekola liphoofolo tsa hao ha kae selemong. Ho na le ho hong hosele!

Frequency	Once a year /Hang selemong	Twice a year /Habeli ka selemo	When necessary /Ha ho hlokahala	Other/ Ho hong ho sele
Tick				

36. Have you increased administration frequency from 5 years ago?

Yes/ee	
No/chee	

37. If yes, What prompted you to increase drug frequency on your flock?/ Ke eng e entseng hore o eketse makhetlo ao o fanang ka moriana ka oona liphoofolong?

Reason	Reoccurrence of symptoms	Recommendation from extension officers	Instinctively	Other/nako e ‘ngoe, hlalosa!
Tick				

38. At what time of the day do you administer anthelmintic drugs/ O sebelisa/fa liphoofolo (linku) tsao moriana ka nako efe ea letsatsi?

Time	Hoseng/ka meso/Pele re bulela	Mantsiboea/ha li kena sakeng	Nako e ‘ngoe feela	Other/nako e nngoe, hlalosa!
Tick				

39. Do you know that drugs drop in efficacy on controlling nematodes? Na o tseba hore matla a moriana a ka theoha ho bolaeeng manyooa?

Yes/Ee	
No/ Chee	

40. Do you suspect any drop in drug efficacy / Ha karabo kaholimo e le ee, see se bakoa keng?

Yes/Ee	
No/ Chee	

41. Do you use alternative remedy (herbal concoctions) to anthelmintics?

Yes/Ee	
No/ Chee	

42. if yes, which ones? /Na o sebelisa mekhoa e meng ea phekolo ea manyooa e kang ea litlama? Ha e be karabo e le ee, o sebelisa eng?

	Moriana/remedy	Mokhoa oa ts'ebeliso/method of application
a.		
b.		
c.		
d.		

43. If yes, under what circumstances do you use an alternative drug? Ha karabo e le ee, ke boemong bofe le makhetlo a makae moriana oo o sebelisoa?

Moriana/remedy	Tick appropriate
As a strategic control to rest Anthelmintic	
Use if every time	
Only when cannot afford anthelmintic	
When anthelmintic cannot be accessed	
Other/specify	

44. What are the benefits of using alternative remedy? Molemo oa ho sebelisa tse ka holimo (40) ke life liphoofolong tsa hao/heno?

a.	
b.	
c.	
d.	

45. Any other comments on the usage of anthelmintics to treat internal parasites in livestock? Ke se fe se seng seo o ka buang ka sona malebana le ts'ebetso le ts'ebeliso ea meriana ea manyooa le melemo ea eona?

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Appendix J: Published journal from thesis.

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Characterization and population genetics of *Haemonchus contortus* in Merino sheep in Lesotho

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ABSTRACT

Haemonchus contortus is the most pathogenic and economically restrictive gastrointestinal nematode in the small ruminant industry globally. Morbidity, poor cross-bodily state, and mortality of sheep in Lesotho suggest the presence of *H. contortus*. The present study investigated the morphological, molecular, and population genetics of *H. contortus* third-stage larvae infecting sheep in four ecological zones (EZ) of Lesotho. Coprocultures were prepared for larval morphological identification and PCR determination. Larvae were identified morphologically as 100% *H. contortus*. The Second Internal Transcribed Spacer (ITS-2) gene of the ribosomal DNA of *H. contortus* isolates in the present study revealed nucleotide homology ranging from 97 to 100% when compared with selected GenBank reference sequences. Pairwise evolutionary divergence among *H. contortus* isolates was low, with 0.01318 recorded as the highest in the present study. Five haplotypes resulted from 14 Lesotho sequences. Haplotype diversity and nucleotide diversity were 0.76923 and 0.00590, respectively. Genetic differentiation among isolates was low but not statistically significant. An analysis of molecular variance revealed that most molecular variation was distributed within topographic populations at 94.79% (F_{ST} = 0.05206, $p > 0.05$) and 5.21% among populations. There was high gene flow and no definite population genetic structure among Lesotho isolates.

1. Introduction

Lesotho is a subtropical country, completely surrounded by South Africa and topographically subdivided into four EZs, namely, the Highlands, Foothills, Lowlands, and the Senqu River Valley (SRV) (Mbata, 2001; Obioha, 2010). Sheep and goats are important constituents of livelihoods for rural populations in Lesotho. Livestock production GDP has been dominated by wool and mohair sales for several decades in Lesotho (Mokhethi et al., 2015). Parasitic infections and substandard husbandry practises are major threats to a successful small ruminant industry across the globe (Dey et al., 2019). Furthermore, the expenses of controlling and managing anthelmintic resistance (AR) in gastrointestinal nematodes (GIN) raise overall economic losses to the small ruminant industry (Iqbal et al., 2005; Munguía-Xóchihua et al., 2018). *Haemonchus* contortus (the barber's pole worm) is the most disreputable GIN, infecting small ruminants. *H. contortus* is mainly prevalent in tropical and subtropical areas, especially the resource-poor livestock farming communities of Africa such as Lesotho (Dey et al., 2019; Lichtenfels et al., 1994; Troell et al., 2006; Murad et al., 2018; Mahlehla et al., 2021). Bisset et al. (2014) observed that grazing livestock are at constant risk for reinfection by GINs. *H. contortus* was recognised as a major cause of anaemic outbreaks characteristic of haemonchosis and is also responsible for overall small ruminant morbidity and mortality around the globe (Angulo-Cubillán et al., 2007; Jabbar et al., 2006; Matebesi-Ranthimo et al., 2014; Papadopoulos et al., 2003; Troell et al., 2006).

Disease diagnosis and pathogen identification are critical components in the determination of infection trends and the conceptualization of daunting epidemiological factors. The importance of correct disease diagnosis cannot be overemphasised because it provides valuable

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information for the identification of disease agents (Molento et al., 2016). Microscopy was successfully used in the laboratory diagnosis of pathogenic nematodes by screening eggs from faecal material and providing a general parasitic viewpoint with limited species identity (Van Wyk and Mayhew, 2013). Comparative morphological identification of adult and larval nematodes to genus and species level has always been useful in laboratory helminth diagnostics (Zarlenga et al., 2016; Heise et al., 1999; Van Wyk and Mayhew, 2013; Matsepe et al., 2021). Morbidity and mortality caused by closely related GINs may require extensive knowledge for accurate differential diagnosis, hence the introduction of more sensitive and species-specific methods of pathogen identification. Morphological identification between *H. placei* parasitizing cattle and *H. contortus* in sheep posed a challenge for precise distinctions, especially where the two hosts co-exist in grazing rangelands (Zarlenga et al., 2016).

Molecular protocols for parasite identification were introduced as additional methods of disease diagnosis that proved effective and more accurate (Emery et al., 2016). Molecular techniques utilize genetic markers to accurately identify all life stages from genus to sub-species level in a single polymerase chain reaction (PCR) assay (Knoll et al., 2021). The most common molecular marker used for intra-specific genetic variation in trichostrongylids is the ITS-2 gene, which has conserved regions and is therefore ideal for precise identity and perfect differentiation between closely related species (Mangkit et al., 2014). Molecular and morphological methods were therefore used concurrently to identify parasites accurately, timely, and effectively for use in various epidemiological determinations (Mangkit et al., 2014). Several studies conducted using microscopy revealed the presence of nematodes, coccidia, and tapeworms as the three main gastrointestinal parasites (GIP) infecting small ruminants in Lesotho (Mahlehla et al., 2021; Moiloa et al., 2020). The present cross-sectional study was conducted to characterise the *H. contortus* third-stage larvae (L3) by morphological and molecular techniques. Furthermore, the population genetics of *H. contortus* from four EZ in Lesotho were investigated using the ITS-2 gene of the ribosomal DNA.

2. Materials and methods

2.1. Study area

The present study is of a cross-sectional type and was carried out in four EZs of Lesotho in December 2021 and January 2022. The study area consisted of 21 collection sites stratified into four EZs, namely, the Highlands, Foothills, Lowlands, and the Senqu River Valley (SRV). Lowlands (1530 m altitude) covered parts of Mafeteng and Maseru districts. Foothills (1800 m in altitude) were located in Leribe district. The Highlands (>2200 m in altitude) were in Thaba Tseka district. SRV (1500–2250 m in altitude) covered areas in Quthing and Qacha'nek districts. The sampled animals were predominantly grazing in communal rangelands. Table 1 presents the EZs, districts, and specific coordinates and names of the respective collection sites within the study area.

2.2. Coproculture preparation and morphomentrical analysis of *H. contortus* L3

Three farms were selected from each of the 21 sampling sites in the four EZs of Lesotho. Ten Merino sheep were randomly selected from each farm, and fecal samples were collected directly from the rectum using surgical gloves. Samples were screened for GIN eggs using the modified McMaster method of fecal egg count (FEC) by Roepstorff and Nansen (1998). For coproculture preparation, fecal samples with a minimum of 500 eggs per gram of feces were considered. These fecal samples from farms within sampling sites were then pooled and coproculture prepared as per Van Wyk and Mayhew (2013) protocol for subsequent morphometrical characterization of L3. The whole mount and STE from five L3s from each suspension from the sampling sites were measured under the compound microscope on a calibrated ocular micrometre. The value of STE was then calculated. Van Wyk and Mayhew (2013) protocol for the identification of the L3 of *H. contortus* in small ruminants calculated an STE with a value of 2.2–2.7 'X' and the equivalent values for *H. placei* in cattle as 2.7–4.0 'X'.

Table 1
Data collections sites depicting ecological zones, districts and accession numbers of ITS-2 sequences submitted to GenBank.

Ecological zone	District	Collection site	Site coordinates	Accession number
Highlands	Thaba Tseka	Mokotane	−29.513912, 28.291366	OQ674238
		Ha Letuka	−29.531186, 28.266342	OQ674239
		Ha Thabo	−29.637831, 28.285523	OQ674240
		Pitseng	−29.008545, 28.211746	OQ674241
		Tsunyane	−29.258623, 28745319	OQ674242
Foothills	Leribe	Matlameng	−28.975891, 28.284456	OQ674243
		Ha Makape	−28.969187, 28.193677	OQ674244
		Mphosong	−29.033452, 28.271869	OQ674245
Lowlands	Maseru	Mokhalinyane	−29.489033, 27.370197	OQ674246
	Mafeteng	Ha Mohlalefi	−29.581655, 27.316686	OQ674248
		Moseletsi	−29.581645, 27.344562	OQ674247
Senqu River Valley	Quthing	Mphaki	−30.195345, 28.167627	OQ674249
		Qhoalinyane	−30.211945, 27.910674	OQ674250
	Qacha's nek	Ha Sekake	−30.120372, 28364839	OQ674251

2.3. Larval DNA extraction

L3s positively identified as *H. contortus* were washed three times in distilled water and three times in phosphate-buffered saline (PBS). Between 5 and 10 L3s were picked from PBS suspensions and placed into 1.5-ml microcentrifuge tubes. The suspensions were then frozen and thawed three times for 10 min at −80 °C and 70 °C, respectively. The L3 suspensions were then centrifuged at 14000 rpm for 10 min to produce pellets. The pellets were manually crushed using an Eppendorf homogenizer and then re-suspended in PBS. The L3 DNA was then extracted from each suspension using the QIAamp DNA mini kit (Qiagen, Germany) as per the manufacturer's instructions. The eluted DNA was stored at −20 °C for PCR assays.

2.4. PCR amplification and sequencing of DNA

PCR amplification of the ITS-2 gene of the ribosomal DNA of *H. contortus* was done using oligonucleotide primers NC1F: 5'-ACGTCTGGTTCAGGGTTGT-3' and NC2R: 5'-TTAGTTTCTTTTCCTCCGCT-3' as described by Stevenson et al. (1995) and Dey et al. (2019). A PCR of 25 µl total volume comprising 10 µl of DNA's free water, 0.25 µl of each primer (10 µM), 12.5 BioRad supermix, and 2.0 µl of DNA template (50–100 ng/µl) was performed. PCR amplification was carried out in an automated BioRad thermocycler (BioRad, USA) as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, with a final elongation step at 72 °C for 7 min. To evaluate the amplification, PCR amplicons were run on 1.5%

gel electrophoresis and measured against a 100 bp ladder. All amplicon bands that measured around 350 bp were considered positive for *H. contortus*. Positive gel bands were column purified using the QIAquick PCR purification kit (Qiagen, Germany), following manufacturer instructions. The purified amplicons' quality and concentration were analyzed by 1.5% gel electrophoresis and NanoDrop (Thermo Scientific, USA). The purified amplicons were sent for Sanger sequencing services in Germany. The consensus sequences were edited and assembled from two complementing forward and reverse sequences using Staden package software, version 2.0.0 (Staden et al., 2000). The generated sequences were subsequently deposited in GenBank and assigned accession numbers (Table 1).

2.5. Data analysis

All sequences in the study were aligned and analyzed for species identity in the BLASTn program of the National Centre for Biotechnology Information (NCBI) (http://www.NCBI.nlm.gov/BLAST). The best substitution model for phylogenetic tree analysis was determined using JModel Test version 2.1.10 software (Darriba et al., 2012). The method entailed selecting the model with the lowest Bayesian information criterion (BIC). The phylogenetic tree with bootstrap test and pairwise alignment of estimates of evolutionary divergence were assembled in MEGA11 software (Tamura et al., 2021). DNAsp version 6.12.03 and Arlequin version 3.5 (Rozas et al., 2017; Excoffier and Lischer, 2010) were used to determine molecular indices comprising nucleotide diversity, haplotype diversity, sequence polymorphism analysis, and neutrality test values. DNAsp version 6.12.03 was also used to calculate the level of allelic dissimilarities within and among parasite populations in the study area. Genetic diversity within and among populations in the four EZ of Lesotho was determined by analysis of molecular variance (AMOVA) using the Arlequin version 3.5 software package (Excoffier and Lischer, 2010). Haplotype network analysis based on a median-joining algorithm signifying the genetic relations between the Lesotho isolate *H. contortus* sequences and 18 other sequences from NCBI was constructed using Popart version 1.7 (Bandelt et al., 1999).

3. Results

3.1. Morphological identification of L3

All prepared coprocultures yielded L3 strongyles. All observed L3s had a characteristic bullet-shaped cranial region. Fig. 1 shows three different aspects of L3 morphology taken under the microscope. The overall length of the L3 was 661.4444 ± 5.18223 μm. The sheath tail extension (STE) measured 81.6944 ± 1.30472 μm and possessed a characteristic kinked filament. The value of STE recorded in the present study was 2.4752 ± 0.03953 'X'. The STE value conforms with that of Van Wyk and Mayhew (2013), confirming the species identified as *H. contortus*. The L3 in the present study were 100% *H. contortus* upon morphological identification, as per Van Wyk and Mayhew (2013).

3.2. Molecular identification of H. contortus L3

The *H. contortus* ITS-2 gene of the ribosomal DNA was analyzed for identity, genetic diversity, and the determination of population structure in the selected four EZs of Lesotho. There were 14 successfully sequenced amplicons in this present study (Table 1). Fig. 2 shows 350 bp single bands of resultant *H. contortus* amplicons on 1.5% agarose gel electrophoresis. The nucleotide homology of all sequences in this study ranged between 97 and 100% when aligned with sequences in the NCBI database. The overall GC content of the sequences in the study was 32.9%. The 231 bp alignment was constructed of 14 ITS-2 gene segment sequences, with accession number X78803 serving as a reference (Stevenson et al., 1995).

The nucleotide analysis of the Lesotho isolate sequences revealed 224 monomorphic sites and six polymorphic sites. There were three singleton variables on nucleotide positions 66, 68, and 79, while the parsimony informative sites had three on nucleotide positions 17, 20, and 201. There were two types of transversion substitution mutations of T/A, the first on nucleotide positions 17, 79, and 201, while the second, C/G, was on nucleotide position 21. The sole transition mutation of the C/T change was on nucleotide position 126. To rule out *H. placei* as a possible identity of Lesotho isolates, isolate sequences were further aligned with the reference sequence, X78812. The alignment revealed three purine transitions at nucleotide positions 24 (G), 205 (A), and 219 (G), which were characterised as species-specific single nucleotide polymorphisms (SNP) for *H. placei* as defined by Stevenson et al. (1995) and Chaudhry et al. (2015). In addition to the species-specific SNPs above, the alignment with both X78812 and X78803 also revealed a common transition mutation (C/T) in all Lesotho isolates at nucleotide position 123.

3.3. Phylogenetic analysis of H. contortus based ITS-2 gene

For the determination of the phylogenetic status of Lesotho *H. contortus* isolates, 18 world sequences from four continents (Africa, Asia, Europe, and Oceania) were selected from NCBI for the analysis. Table 2 presents global *H. contortus* ITS-2 sequences from NCBI used in the inferred phylogenetic tree with Lesotho isolates. Fig. 3 presents the 32-sequence Maximum Likelihood Tree constructed in the MEGA 11 program (Kimura, 1980). The phylogenetic analysis of Lesotho isolates with 18 other global sequences was based on 231 bp of the ITS-2 gene region of the ribosomal DNA of *H. contortus*. The tree was rooted with the ITS-2 partial gene sequence of *Trichostrongylus colubriformis* (accession number: AB90896) as an outgroup. The phylogenetic tree was divided into three major clades (A, B, and C). Clade was the largest

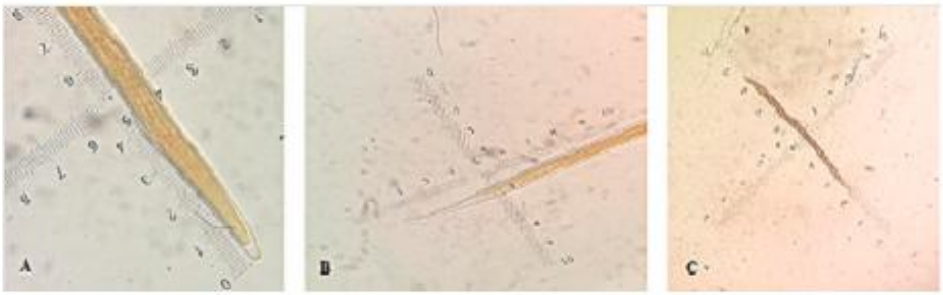

Fig. 1. Photomicrograph of *H. contortus* L3: A- the bullet-shaped head/cranial, (X40), B- sheath tail extension caudal end (X40) and C- whole mount (X10).

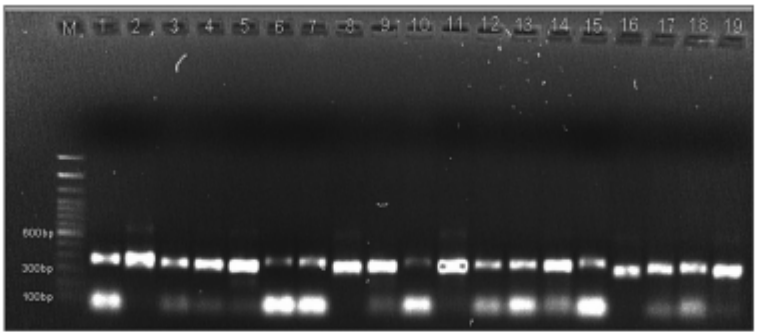

Fig. 2. The *Haemonchus contortus* larval DNA amplicons with PCR product of ±350 bp. A ladder of 100 bp was used. Lane1 = Mokotane, Lane 2 = Ha Letuka, Lane 3 = Ha Leronti, Lane 4 = Ha Thabo, Lane 5 = Macheseng, Lane 6 = Pitseng, Lane 7 = Tsunyane, Lane 8 = Matlameng, Lane 9 = Ha Makape, Lane 10 = Mphosong, Lane 11 = Ntsie, Lane 12 = Ha Makintane, Lane 13 = Ha Khola, Lane 14 = Matsoseng, Lane 15 = Ha Mohlalefi, Lane 16 = Ha Mokhalinyane, Lane 17 = Ha Moeletsi, Lane 18 = Hermoni, Lane 19 = Mphaki.

Table 2
Global ITS-2 gene sequences based on 231 bp from the GenBank used for phylogenetic analysis and haplotype network analysis with Lesotho isolates in the present study. Sequences are presented with their accession numbers, host species, life cycle stage, country of origin and haplotype number in the haplotype network analysis.

	GenBank accession number	Host species	Life cycle stage	Country of origin	No. of haplotypes
1.	X78803	–	adult/ adult/ egg	Australia	16
2.	MT193663	Goat	Larval	China	1
3.	AB908961	Goat	Adult	Japan	1
4.	KC998713	Sheep	Larvae	New Zealand	6
5.	KC998714	Sheep	Larval	New Zealand	1
6.	AB682686	Sheep	Adult	Egypt	9
7.	MT568605	Sheep	Larval	Myanmar	11
8.	LC368060	Goat	Adult	Nigeria	14
9.	MN708983	Sheep	Adult	Cameroon	14
10.	MH481595	Cattle	Larval	Ghana	14
11.	AB682687	Sheep	Adult	Egypt	10
12.	KP760874	Blue Wildebeest	–	Kenya	17
13.	JF680983	Sheep	Adult	Ireland	8
14.	AB908963	Goat	Adult	Laos	12
15.	KM586652	–	Larval	China	13
16.	LC360154	Goat	–	Bangladesh	15
17.	KX829170	Cow	–	Iran	7
18.	AB908660, *T. Columbriformis*	Goat	Adult	Laos	–

group, bearing a total of 28 sequences in smaller sub-clades. All Lesotho isolate sequences were in clade A. Clade B is comprised of three sequences from New Zealand (KC998713), Ireland (JF680983), and Laos (AB908963). All *H. contortus* sequences portray a possible single ancestry for clades A and B.

A pairwise alignment representing estimates of evolutionary divergence between five haplotypes derived from 14*H. contortus* sequences from different EZs of Lesotho is shown in Table 3. The pairwise evolutionary divergence revealed very low variability (0.0000–0.01318) in the ITS-2 gene. The pairwise nucleotide diversity was low across the Lesotho populations. The highest pairwise value (0.01318) was between the SRV isolate and six other isolates from the Highlands, Lowlands, and Foothills (OQ674238, OQ674239, OQ674240, OQ674246, OQ674244, and OQ674251).

3.4. Genetic diversity and population genetics of *H. contortus* in Lesotho

The Lesotho isolates analyzed in DNAsp v6 and Arlequin 3.5.18 revealed five distinctive haplotypes (Librado and Rozas, 2009; Excoffier and Lischer, 2010). The molecular indices depicting the intra-population dynamics of the Lesotho isolates for four EZs are presented in Table 4. The average haplotype diversity (HD) and nucleotide diversity (Pi) values in the present study were 0.76923 and 0.00590, respectively. The Lowlands and SRV haplotype diversity of 1.0 symbolised that all sequences from these EZ had distinct sub-populations, hence different haplotypes. Highlands had a haplotype diversity of zero, which displays complete homology and a singleton haplotype.

Fig. 4 represents the haplotype network analysis of Lesotho isolates and selected sequences from four continents (Africa, Asia, Europe, and Oceania). The presentation also provides haplotype frequencies and the number of mutations between respective haplotypes. Haplotypes 1 and 2 were the most common, with eight and five members, respectively. Haplotype 1 is comprised of five Lesotho isolates (OQ674238, OQ674239, OQ674240, OQ674244, and OQ674251), two from Asia (MT193663 and AB908961), and one from Oceania (KC998714). Haplotype 2 consisted of Lesotho isolates only, which included isolates from the foothills (OQ674241, OQ674243, and OQ674245), SRV (OQ674248), and lowlands (OQ674247). The sequences OQ674242 and OQ674250 belonged to haplotype 3 and were from the Foothills and SRV, respectively. Haplotypes 4 and 5, both from the Lowlands, were singular and belonged to OQ674246 and OQ674248, respectively. Haplotypes 6 to 17 belonged to selected GenBank sequences from different continents, as portrayed in Table 2. There seemed to be few mutations between Lesotho isolates, as depicted in the haplotype analysis network. Lesotho Haplotype 4 was the most distant from Asia's Haplotypes 12 and 13, with a total difference of 10 mutations between them.

Genetic differentiation of *H. contortus* L3 isolates among EZ populations was established through pairwise F_{ST} values (Table 5). The highest inter-population genetic differentiation (F_{ST} = 0.38776) was recorded between pairwise comparisons between the Highlands and Foothills, while the least genetic dissimilarity (F_{ST} = −0.22727) was recorded between the Lowlands and the SRV. The negative pairwise F_{ST} values are regarded as the same as zero. The F_{ST} values between the Foothills, Lowlands, and SRV are all negative. The values for Tajima's D and Fu's Fs tests were − 0.24314 and 0.01278, respectively. The neutrality tests revealed there was no significant deviation ($P > 0.05$) from neutrality within the parasite sub-population. An analysis of molecular variance (AMOVA) was calculated to determine gene flow within and among EZ populations in Lesotho. The majority of molecular

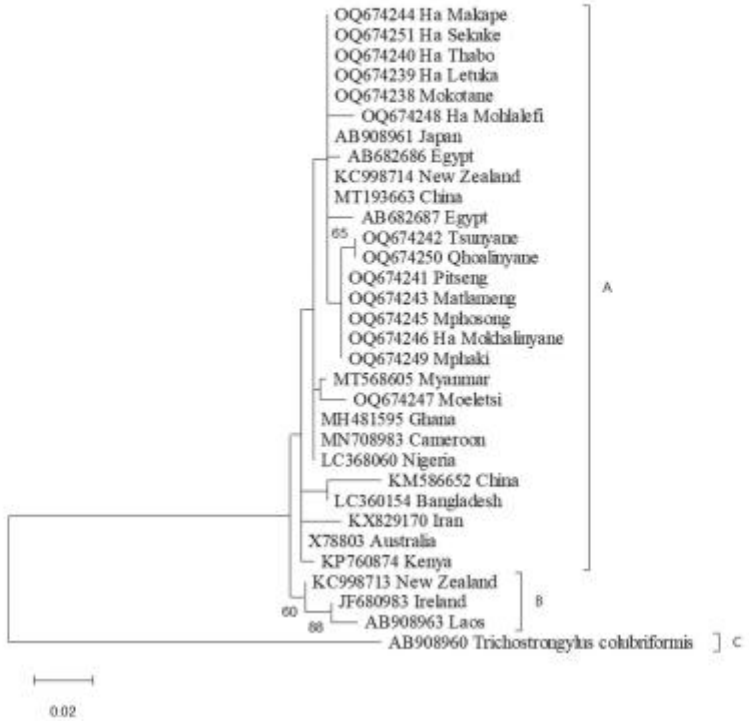

Fig. 3. Phylogenetic relationship of *H. contortus* Lesotho isolates inferred from IST-2 of the ribosomal gene from and 18 other publicly available across the globe. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) represents the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated. There were a total of 224 positions in the final dataset (Tamura et al., 2021).

Table 3
Estimates of evolutionary divergence between five haplotypes of *H. contortus* based on ITS-2 sequences from four ecological zones of Lesotho.

Haplotypes	Hap 1	Hap2	Hap 3	Hap 4	Hap 5
Hap 1	0.0000				
Hap2	0.00436	0.0000			
Hap 3	**0.01318**	0.00436	0.0000		
Hap 4	0.00000	0.00876	0.00876	0.0000	
Hap 5	0.00876	0.00876	0.00876	0.00876	0.0000

variation had a distribution within topographic populations of 94.35%, while 5.65% was among EZ populations ($F_{ST} = 0.05651$, $p > 0.05$).

4. Discussion

The L3 identification is essential for the epidemiological determination of helminthiasis and provides information on the pathogenicity and transmissibility of GIN within the host population. Even though the identification principle has not changed extensively over several decades, Van Wyk and Mayhew (2013) provided a practical approach to the identity of GINs' L3 through a comparative use of morphological features. Cultured L3s from all four EZs were identified with a bullet-shaped anterior end. Van Wyk et al. (2004) recorded an average STE

Table 4
Molecular indices of *H. contortus* populations based on the partial segment of the ITS-2 of the ribosomal gene from four ecological zones of Lesotho.

EZ	N	NPS	H	SS	HD	Pi	MNPD	SSF	Tajima's D	Fu's Fs
Highlands	3	0	1	0	0.0	0.0	0.0	1.00	0.000	0.000
Foothills	5	4	3	2	0.700 ± 0.2184	0.00774 ± 0.06202	1.800 ± 1.236	0.440	−0.926	0.469
Lowlands	3	5	3	2	1.000 ± 0.2722	0.01425 ± 0.01235	3.333 ± 2.323	0.307	0.000	1.000
SRV	3	4	3	3	1.000 ± 0.2722	0.01149 ± 0.10316	2.667 ± 1.919	0.333	0.000	1.000
MTt	14	6	5	7	0.7692	0.00590	–	–	−0.2431	0.0128

NB: EZ = ecological zone, SRV=Senqu river valley, N = Number of sequences, NPS=Number of polymorphic sites, H=Number of haplotypes, SS = sites with substitutions HD=Haplotype diversity, Pi = Nucleotide diversity, MNPD = Mean no. pairwise differences, SSF = Sum of square frequencies, MTt = mean total, TsD = Tajima's D value, Fu's Fs = Fu's Fs *neutrality test*.

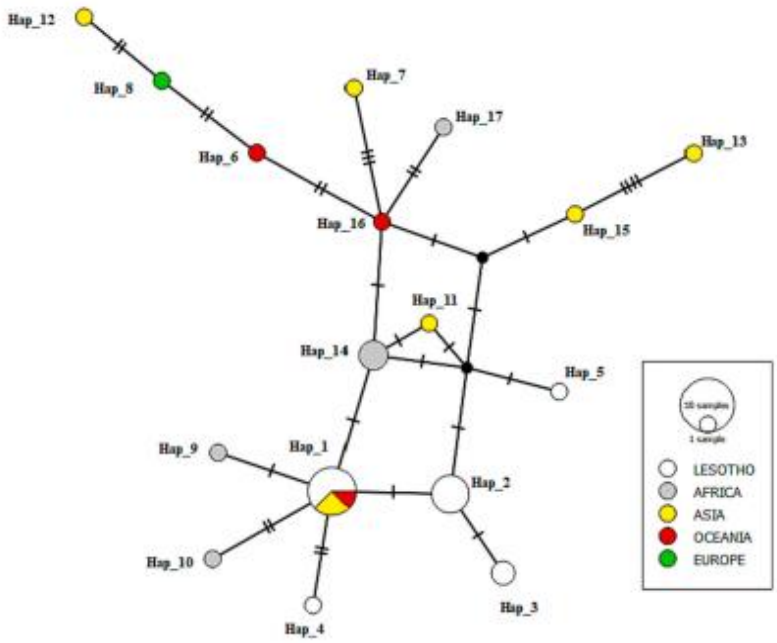

Fig. 4. Median-joining haplotype analysis of Lesotho and selected global *H. contortus* based on ITS-2 partial gene (231 bp) sequences. 17 haplotypes resulted from the overall 31 sequences. Each disc represents a haplotype; the disc size depicts relative frequencies for each haplotype. Different color shades represent either continental and/or Lesotho populations. Mutation changes are represented by lines between the discs (Bandelt et al., 1999).

Table 5
Population pairwise F_{ST} values of *H. contortus* L3 from four AEZs of different EC of Lesotho based on ITS-2 of ribosomal DNA.

Ecological zone	Highlands	Foothills	Lowlands	SRV*
Highland	0.0000	–	–	–
Foothills	0.38776	0.00000	–	–
Lowlands	0.16667	−0.05192	0.00000	–
SRV*	0.33333	−0.21238	−0.22727	0.00000

* Senqu River Valley.

length of 74 μm and estimated the 'X' value for *H. contortus* to range between 2.2 and 2.7. 'X' is a comparative value calculated from an equation involving the usage of the STE of Trichostrongylus columbriformis for relativity. Moudgil et al. (2022) measurements of *H. contortus* yielded a STE of 71.9 ± 2.8 μm. Though the STE average length of *H. contortus* in the present study was relatively longer, the calculated 'X' value fell within the range prescribed by Van Wyk and Mayhew (2013). The length of STE did not show any statistical difference ($p > 0.05$) between EZ populations in the present study. Matsepe et al. (2021) reported *H. contortus* as the sole strongyle infecting goats in Lesotho, which is similar to our results in sheep. Abu-Elwafa et al. (2016) study on identification of nematodes L3 of ruminants revealed *H. contortus* L3 from sheep as medium-sized, measuring 680–800 μm, bullet-headed, and with a kinked STE of 68–80 μm. Similar morphological features, such as a bullet-shaped anterior and kinked tail, were recorded by Veena et al. (2020). Differential diagnosis ruled *H. placei* out due to its comparatively longer mean STE (102 μm) and higher range (80-119 μm) from our result. Similarly, *Cooperia* has a somewhat similar STE range to *H. contortus*, except for the difference in the overall length of the L3 and the shape of the 'head' region. The result offered reasonable confidence in the affirmative identity of *H. contortus* in the present study. Amarante (2011) emphasised the importance of correct identification and differentiation of sympatric *Haemonchus* species for collecting epidemiological data for subsequent responses in parasite management and control.

The present molecular work on L3 of *H. contortus* based on the ITS-2 gene was the first in Lesotho. Molecular identification and characterization have gained momentum in recent years as accurate protocols in diagnostics, as also demonstrated in our study. The method provided the requisite information in parasitology and epidemiology, consequently facilitating tracer studies in phylogenetics and genetic diversity (Yin et al., 2016). The gel electrophoresis band size of the amplicon in the study was consistent with findings in Laosutthipong and Eardmusic (2019), Kandil et al. (2017), and Stevenson et al. (1995). The average GC content of ITS-2 in the present study was 32.9%, while Mangkit et al. (2014) and Laosutthipong and Eardmusic (2019) reported 32.9% and 34.1%, respectively. The high homology and single open reading frame observed in the comparisons between ITS-2 of the Lesotho isolates and X78803 confirm origin from one ancestor. A similar molecular marker analysis was employed by several scholars across the globe to study *H. contortus* identification and population genetics (Australia, Brazil, China, Italy, Malaysia, Pakistan, the USA, and Yamen) (Yin et al., 2016). Hade et al. (2022) reported that all isolates in their Iraqi survey were identified as *H. contortus*. The ITS-2 intra-specific variation in our study was comparable to the result found by Mangkit et al. (2014) of <1%. Stevenson et al. (1995) did not find any variation among 10 *H. contortus* DNA isolates of identical gene segments in Australia. The highest percentage variation in the present survey was 1.318% among Lesotho isolates. The study findings are consistent with Yin et al. (2013) findings,

where intraspecific variation of 2.6% in 152 ITS-2*H. contortus* was recorded from different regions in China. The multiple alignment comparison of Lesotho isolates with reference sequence accession numbers X78803 and X78812 revealed three purine transitions at nucleotide positions 24 (A), 205 (G), and 219 (G), with the latter therefore confirming the identity of *H. contortus* as reported by Laosutthipong and Eardmusic (2019), Pitaksakulrat et al. (2021), and Stevenson et al. (1995).

The analysis of the nucleotide sequences from Lesotho isolates produced a single open reading frame of ITS-2 of the nuclear ribosomal DNA among themselves and *H. contortus* ITS-2 gene sequences retrieved from GenBank. The overall haplotype diversity (0.76923) recorded in the study is well within the range (0.609 to 0.824) recorded by Yin et al. (2013) using ITS-2. Our results were relatively lower than those recorded by Dey et al. (2019) in Bangladesh, Qamar et al. (2022) in Pakistan, and Laosutthipong and Eardmusic (2019) in Thailand, who reported haplotype diversity of 0.8695, 0.846, and 0.832, respectively. Kandil et al. (2017) epidemiological study of genetic diversity and patterns of gene flow for *Haemonchus* species in small ruminants revealed that PCR and gene sequencing led to accurate taxonomic classification of different parasite genotypes. On the one hand, the average nucleotide diversity (0.00590) for Lesotho isolates was lower than results from Bangladesh (0.0098), Thailand (0.017), and Pakistan (0.0214).

The high values of haplotype diversity suggest a high probability of intra-genetic diversity in Lesotho *H. contortus* subpopulations. That being said, the haplotype diversities in a study by Shen et al. (2017) on *H. contortus* from domestic sheep were lower (0.719) than the present results, while those of wild blue sheep (0.787) from China were similar to our results. The zero haplotype diversity value for *H. contortus* of the Lesotho Highlands was in principle comparable to work by Stevenson et al. (1995), which did not yield any variation among 10*H. contortus* DNA isolates on ITS-2 in their study in Australia. Nucleotide diversity in the present study was lower than results by Dey et al. (2019) and Laosutthipong and Eardmusic (2019), recording 0.0098 and 0.017, respectively. The study by Qamar et al. (2022) recorded relatively higher nucleotide diversity (0.0214) than the present study. Inversely, the study by Shen et al. (2017) recorded relatively lower nucleotide diversity in domestic sheep (0.004) but lower than recorded for wild blue sheep (0.008). The recorded nucleotide diversity value in Lesotho depicts little average pairwise genetic diversity among all sampled isolate sequences in our study. This was supported by estimates of evolutionary divergence through a pairwise alignment analysis, which resulted in low intra-specific genetic variation. All Lesotho sequences with zero values on the pairwise estimates for evolutionary divergence clustered on the phylogenetic tree. The characteristic low genetic variation in ITS-2 also seen in this study supports the use of the gene segment in species identification. Consequently, the molecular marker continues to be an ideal instrument for differentiation between very closely related species and even at the sub-species level. Low genetic variation was also recorded by Hade et al. (2022) in *H. contortus* in Iraq on a similar molecular marker.

There was a similar trend of ITS-2 haplotypes in the present study to some reported elsewhere. We recorded five haplotypes from 14 sequences, while Blouin et al. (1995) reported 113 haplotypes in the overall 120 sequences, and Dey et al. (2019) recorded 77 haplotypes out of 85 sequences. The result in the present study corroborated Pitaksakulrat et al. (2021), who also reported an insignificant display of geographical genetic structure among *H. contortus* isolates. The present results demonstrated an apparent interrelationship between the pairwise divergence values, phylogenetic tree analysis results, and haplotype analysis.

Haplotypes 1, 2, and 3 were shared and seemed to traverse ecological delineations. Factors responsible for the genetic diversity and population genetic structure of parasites include distribution by host movements, population size, environmental and geographic conditions, life history, and gene flow (Gilleard and Redman, 2016). The common haplotype for the Highlands isolates may be attributed to transhumance, which congregates animals in the same pastures, promoting gene flow. Invariantly, rangelands in the lowlands are divided according to village jurisdiction (Mbata, 2001), where different haplotypes were recorded. However, our findings might be influenced by the small population size.

The evolutionary divergence disparity between Lesotho isolates and those from other countries and continents may be due to allopatry, a population structure of separation by distance. The present study strongly supports the suggestion by Dey et al. (2019) that there are two distinct global geographical patterns of *H. contortus* clades influenced by continental isolation. Blouin et al. (1995) also suggested that the population genetic structure of other GINs depicted major diversity distributed within populations, as evident in the present study.

5. Conclusion

In conclusion, the results showed that all L3 identified morphologically in the present study were *H. contortus*. The morphological identification was confirmed by molecular protocols showing clear single bands characteristic of the *H. contortus* ITS-2 full gene. Morphology and molecular protocols can therefore be used effectively at different levels for specific laboratory diagnosis needs. The population indices in our study were in unison in establishing a more homogeneous population of *H. contortus* in Lesotho, regardless of different EZs and different village grazing delineations. Divergent evolution and mutation is suspected as a for variation between Lesotho isolates and those from other continents. The results of the present study provide valuable insights into the patterns of parasite gene flow, which may be used in formulating parasite control strategies and baseline for further research.

Ethical approval

The postgraduate research committee of the Faculty of Agriculture approved the research project. The study samples were sourced from animals using the standard laboratory diagnostic procedures for faecal collection and examination protocols. Animals were not used as experimental units. The Lesotho Wool and Mohair Growers Association ratified the implementation of the project. The feces were collected with prior agreement with the farm owners.

Authors' contributions

MSP and PMD conceptualized and planned the study. MSP, PMD, and MJRM designed the methodology of the study. MSP and MJRM did field work and collected data. MSP, OMMT, LB, and MJRM designed and executed the laboratory analysis. ACA and MSP conceptualized data analysis, and MSP, ACA, LB, and PMD analyzed the data and made statistical inferences. MSP drafted the manuscript. All authors read, reviewed, and approved the final manuscript.

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