Gastrointestinal nematodes infecting sheep in Limpopo province: Seasonal prevalence and anthelmintic resistance

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DEDICATION

This thesis is dedicated to my wonderful wife Mapitso Mphahlele and my two amazing boys, Olerato and Mosa Mphahlele. You guys are my inspiration to wake up every morning and face the day.

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I thank my Lord and savior Jesus Christ for the opportunity to study and advance in life. I believe His unchanging and eternal word concerning my life as it is written in Jeremiah 29:11 (KJV 2000) “For I know the thoughts that I think towards you, says the LORD, thoughts of peace, and not of evil, to give you an expected end”. Thank you, Lord, that in you I live and move and have my being (Acts 17: 28). Lord you have been faithful!!

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GENERAL ABSTRACT

The annual cost associated with treatment of parasitic diseases in small ruminants is estimated to tens of billions of US dollars worldwide, from the sales of anthelmintic drugs by pharmaceutical companies, excluding production losses. In small ruminants, gastrointestinal nematodes (GINs) can result in anaemia due to the blood-sucking activities of some nematodes species which impact negatively on the profitability of the farm. Objectives of this study were to determine the seasonal occurrence of gastrointestinal nematodes (GINs) of sheep of of resource-poor farmers in Limpopo province of South Africa, risk factors associated with anthelmintic resistance (AR) and to assess the efficacy of most commonly used anthelmintics. Furthermore, to determine the phylogenetic position and genetic diversity of the most pathogenetic nematode species of sheep, *Haemonchus contortus* isolated from sheep in the Limpopo province.

The study was conducted in five districts of the Limpopo province, namely Capricorn, Sekhukhune, Waterberg, Vhembe and Mopani for a period of 21 months. To determine the seasonal prevalence of GINs faecal samples were collected from 156 sheep in each district. They were analysed using the McMaster technique to determine faecal egg counts and faecal cultures were prepared for nematode identification. FAMACHA© was used to assess anaemia in study animals and monthly climate data were acquired from South African Weather Services (SAWS). A structured questionnaire with a combination of qualitative and quantitative, open-ended questions was administered to 77 sheep farmers in Limpopo province of South Africa to evaluate their knowledge on the use of anthelmintics. To determine anthelmintic resistance (AR) in GINs of sheep both in *vivo* and *in vitro* techniques were used. Forty sheep from flocks with high treatment frequencies from each of the five districts were divided into three treated groups and one untreated control group. Group 1 was treated subcutaneously with ivermectin (Ivomec®, Merial, 0.2 mg/kg bw), group 2 was orally dosed with levamisole (Tramisol Ultra®, Coopers and Intervet, 5 mg/kg bw) and the third group was orally dosed with albendazole (Valbazen®, Pfizer, 7.5 mg/kg bw). Group 4 represented the untreated control.

Egg hatch assay (EHA) was used to determine AR against thiabendazole (TBZ) and micro-argar larval development test (MALDT) was used for both TBZ and levamisole (LEV). Phylogenetic position as well as genetic diversity of *Haemonchus contortus* isolated from naturally infected sheep in Limpopo Province, South Africa, in relation to the worldwide populations, was determined using the ITS2 gene region to amplify 259 bp DNA fragment using species-specific primers. Data were analysed using Statistical Analysis System (SAS).

A high nematode prevalence ranging from 88 to 99% was recorded in all districts. During the cold dry season, prevalence decreased to a range between 75 and 83%. However, the observed decrease in egg per gram of faeces (EPG's) during the cold dry season did not differ significantly ($p > 0.05$) among the districts except for Mopani and Vhembe districts. *Haemonchus contortus* was the most dominant nematode species (70 – 93%) in all the districts followed by *Trichostrongylus/Teladorsagia* spp. (5-28%) and *Oesophagostomum columbianum* (<5%) An increase in FAMACHA$^{©}$ scores was recorded when the FEC increased, resulting in a positive correlation ($r = 0.959$; $p = \leq 0.01$). The most common risk factor associated with the occurrence of AR in all the five districts of was the use of anthelmintics without weighing the animals to determine the correct dosage band. Limited farming experience was also shown as one of the risks. Although 67.5% of farmers mentioned that they never dose their sheep, 32.5% used anthelmintics at varying times of the year. A strong correlation existed between faecal egg count reduction test (FECRT) and EHA as both tests confirmed the existence of AR for the tested anthelmintics in all the districts except for LEV in Sekhukhune. *Haemonchus contortus* was the most dominant resistant nematode speciesidentified. No polymorphism was observed within *H. contortus* isolates from Limpopo province. Phylogenetic analyses revealed four major lineages. Limpopo isolates shared common ancestry with reference sequences from Africa, as well as across the globe. The only isolates that did not cluster with the South African isolates were from the USA. Low levels of structure observed in the present study among our isolates and from elsewhere could imply a high level of gene flow.

Seasonal pattern of GINs observed in this study have shown that climate change has not affected the seasonality of nematodes as the results compares with previous studies on nematode seasonality. Occurrence of AR and risk factors associated with AR Limpopo province suggest that there is a need to train rural resource-poor livestock farmers on proper use of anthelmintic treatment and to educate them on methods to prevent development of AR in their flocks. The findings of this study provide a basis for future studies in understanding and controlling the spread of anthelmintic resistance against *H.*

contortus and other GINs in sheep in Limpopo province and South Africa at large and also for tracing changes in the population genetic structure of *H. contortus* in Limpopo.

Keywords: Seasonal prevalence; FAMACHA$^{©}$; anthelmintic resistance; visual appraisal; ITS2 gene; genetic diversity; gene flow; haplotype; Limpopo province; South Africa.

RESEARCH OUTPUTS

Full-length article

Morutse Mphahlele, Ana M. Tsotetsi-Khambule, Rebone Moerane, Majela L. Mashiloane and Oriel M.M. Thekisoe (2018). Risk factors associated with occurrence of anthelmintic resistance in sheep of resource-poor farmers in Limpopo province, South Africa. Tropical animal Health and Production. 51(3): 555-563. https://doi.org/10.1007/s11250-018-1724-2.

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Conference papers

Morutse Mphahlele, Lehlohonolo Mofokeng, Bridget Makhahlela, Siphamandla Lamula, Ana M. Tsotetsi-Khambule, Rebone Moerane, Metlholo A. Phukuntsi, Moeti O. Taioe and Oriel M.M. Thekisoe. Population genetic structure of *Haemonchus contortus* in Limpopo Province, South Africa: a preliminary study. 15 - 17 September 2019, 48th Annual PARSA conference, Safaris Hotel, Windhoek, Namibia.

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Morutse Mphahlele, Ana M. Tsotetsi-Khambule, Rebone Moerane, Dennis Komape, Oriel M.M. Thekisoe. Anthelmintic resistance of gastrointestinal nematodes of sheep in Limpopo province, South Africa. 16 - 18 September 2018, 47th Annual PARSA conference, Tshipise Forever Resort, Limpopo Province, South Africa.

Morutse Mphahlele, Ana M. Tsotetsi-Khambule, Rebone Moerane, Oriel M.M. Thekisoe. Risk factors associated with occurrence of anthelmintic resistance in sheep of resource poor farmers in Limpopo province, South Africa. 16 - 18 September 2018, 47th Annual PARSA conference, Tshipise Forever Resort, Limpopo Province, South Africa.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background

Livestock mortality due to gastrointestinal nematode infections is common in tropical and subtropical regions, where marginal levels of nutrition exacerbate the detrimental effects of infection (Ademola and Eloff 2010). As a result, gastrointestinal nematodes constitute a limiting factor to small stock production and food security (Kemper et al. 2009). Three classes of helminths are distinguished, namely nematodes (roundworms), cestodes (tapeworms) and trematodes (flukes) (Raza et al. 2014).

Gastrointestinal nematodes (GINs) are major parasites with a number of species infecting both cattle and small ruminants (Bricarello et al. 2007). In the tropical and sub-tropical regions of the world, GINs are known to be the most important group of parasites. The main species in cattle include *Haemonchus placei, Cooperia* spp. and *Oesophagostomum radiatum* (Neves et al. 2014). In the case of small ruminants, *Haemonchus contortus, Trichostrongylus colubriformis* and *Oesophagostomum columbianum* are the most economically important GINs (Amarante 2013). Several other species can also occur in ruminants with *Strongyloides* spp. and *Trichuris* spp. being the most common nematodes that present a worldwide distribution. In addition, other species like *Ostertagia ostertagi* and *Teladorsagia cicumcincta* occur in cattle and small ruminants respectively (Knight 2015).

The prevalence of GINs is mostly guided by factors such as relationship between crop adaptation and climate conditions like quantity and quality of pasture, temperature, humidity and grazing behaviour of the host (Pal and Qayyum 1993). During the hot wet months of the year, environmental conditions are conducive for the development of, gastrointestinal parasites and they multiply rapidly with a subsequent high intensity. The perfect temperature range ideal for larval development of many nematode species in the microclimate of the pasture lies between 22 and 26°C while the optimal humidity is close to 100% (Getachew et al. 2007). Most larvae die during unfavourable conditions due to hot or cold climates (Gadahi et al. 2009).

Seasonal dynamics of nematode infections are the result of inter-relationships between the small ruminants, their husbandry and the prevailing climate. The patterns of pasture contamination by nematode eggs and their larvae are mainly similar throughout the year (Vlassoff et al. 2001). The number of infective larvae builds up on pasture over the hot wet summer months to reach a peak in autumn/early winter (Roeber et al 2013).

Limpopo province provides a perfect climate for gastrointestinal nematodes to thrive because it is hot from October to March, with average temperatures rising to 27ºC and decreasing to 20ºC in winter. The bulk of the precipitation occurs in summer, and annual rainfall ranges from about 400 - 600 mm over most of the province (Anon 2007). Most of the population is rural based with a high number of rural dwellers dependent on natural resources and livestock and crop farming (Thomas et al. 2007). Limpopo province is one of the developing provinces in South Africa and is particularly vulnerable to climate change impacts, due to its exposure to extreme weather events (Cook et al. 2004). The province experiences long sunny days and dry weather conditions on most days of the year. During the summer months, which extends from November to January, warm days are often interrupted by short-lived thunderstorms (Limpopo Department of Agriculture 2008).

A variety of pathogens and disease conditions may be influenced by climate change in years to come (Hristov et al. 2018). Foreseeing climate-driven changes in the seasonal availability of free-living gastrointestinal nematode infective stages is the first step to get the measure of the potential impact of climate change on nematode infections in livestock and developing sustainable strategies to control gastrointestinal nematodes (Rose et al. 2015). Certain elements of worm control strategies may serve to select for resistance, in particular the timing of dosing for the various parasitic infections. This aspect is potentially further complicated by the effects of climate change on parasite epidemiology (McMahona et al. 2012).

The primary means of controlling GIN infections in livestock employed by South African farmers is the use of anthelmintic drugs primarily ivermectin, albendazole and levamisole (Tsotetsi et al. 2013). The consequence of inappropriate anthelmintic treatment procedures (e.g. poor quality drugs, poor dosing procedures, intensive use of anthelmintics, etc.), has resulted in the development of resistance to the three classes of broad-spectrum anthelmintic drugs (benzimidazoles, imidothiazoles and macrocyclic lactones) in countries that rear large small ruminant populations (Kaplan 2004; Coles 2005). Despite farmers employing various methods to combat AR, there are reports of decrease in efficacy (Tsotetsi et al*.* 2013). Anthelmintic resistance is defined as a decrease in the efficacy of an anthelmintic against a population of parasites that is generally susceptible to that drug (Sherill et al. 2006). This decrease in susceptibility is caused by an increase in the frequencies of ‘‘resistance’’ gene alleles that result from selection through repeated use of an anthelmintic. Gastrointestinal nematodes of small ruminants have a number of genetic characteristics that promote the development of AR. Among the most important of these features are: (1) rapid rates of nucleotide sequence evolution and extremely large populations resulting from the high fecundity of each individual nematode, providing an exceptionally high level of genetic diversity and (2) a population structure consistent with high levels of gene flow (dissemination), suggesting that host movement is an important determinant of nematode population genetic structure. As a result, these helminths have the genetic potential to respond rapidly and successfully to chemical attack and the means to ensure dissemination of their resistant genes by host movement from farm to farm (Sherill et al. 2006).

In South Africa, AR was reported for the first time in sheep in 1975 and the severity of resistance has increased rapidly ever since (Van Wyk et al. 1997a). Nematodes are becoming resistant to available anthelmintics faster than new anthelmintics are being produced and no reversion of susceptibility seems to have occurred (Van Wyk et al. 1997a). Multi-drug resistant nematodes, of which some are resistant to all classes of anthelmintics, are found worldwide (Kaminsky et al. 2008). This rapid development of multi-drug resistance emphasizes the need to develop new classes of anthelmintics (McKellar and Jackson 2004; Kaminsky et al. 2008). Bath (2014) suggested that apart from developing new anthelmintics, new holistic solutions must also be investigated to avoid an overreliance on anthelmintic drugs. By doing this, nematode populations and infections may be controlled in a way that also prolongs the useful lifespan of future anthelmintics.

It is against this backdrop that accurate identification and genetic characterization of GINs have significant practical implications for the control of nematodes in livestock (Gasser et al. 2008). Moreover, although routinely used in most parasitology diagnostic laboratories, the technique of larval culture coupled with larval differentiation by microscopy is time consuming, laborious to perform, sometimes inaccurate and cannot be readily automated (Roeber et al. 2013). It is these kind of limitations that make molecular studies using genetic markers (e.g. mitochondrial and nuclear DNA) to depict geographical movements of parasitic nematodes to be valuable (Archie and Ezenwa 2011). A number of PCR assays have been developed for the identification or differentiation of strongylid eggs or larvae, utilising genetic markers in the first and second internal transcribed spacers (ITS1 and ITS2, respectively) or external transcribed spacer (ETS) of nuclear ribosomal DNA (rDNA) (Bott et al. 2009).

1.2 Statement of the problem

Previously Van Wyk (1999) recorded the prevalence of GINs in Limpopo province, however, the study did not address the seasonal patterns of infection and did not cover all districts of Limpopo province, namely; Capricorn, Sekhukhune, Waterberg, Mopani and Vhembe. It is therefore, important to provide up to date information on the prevalence and seasonal occurrence data of GINs in all districts of Limpopo province.

The control of parasitic disease in livestock relies on strategic dosing with anthelmintics. Frequent and often excessive use of these drugs has led to widespread problems with AR in parasites of livestock (Taylor et al. 2002). This is exacerbated by the fact that genes conferring anthelmintic resistance are thought to be present in a small portion of individuals in the population even before the worms are exposed to a drug for the first time (Jackson and Coop 2000). Anthelmintic resistance to the three most widely used classes of anthelmintic drugs (benzimidazoles, imidazothiazoles and macrocyclic lactones), is now widespread and resistance to the two newer classes, namely; the amino-acetonitrile derivatives (AADs) and paraherquamide derivatives, is expected to follow (Kaminsky et al. 2008). According to Vatta and Lindberg (2006), AR has been reported throughout Africa, being a particularly serious problem in South Africa and to a lesser extent in Kenya. In South Africa, AR in the commercial sheep farming sector has been described as being the worst in the world (Vatta and Lindberg 2006). In resource-poor livestock farming systems in South Africa, resistance has been reported in sheep in one study and in goats in another two studies (Bakunzi 2003; Tsotetsi et al. 2013). Nematodes resistant to benzimidazoles (i.e. albendazole, thiabendazole, fenbendazole), imidazothiazoles (i.e. levamisole), and macrolides (i.e. ivermectin) have been reported in almost all continents; wherever livestock are regularly treated with anthelmintics (Prichard 1994). It is important to determine the existance of AR in GINs of sheep reared by rural resource-poor farmers, in all districts of Limpopo province.

On the other hand, it is a well-documented fact that *H. contortus* is one of the most successful and problematic livestock parasites worldwide (Gilleard and Redman 2016).

In a study conducted by van Wyk 1999, differential larval counts indicated such a predominance of the genus *Haemonchus* (>95%) in every sheep farm that was surveyed and as a result, only the results of this worm genus were discussed in their paper. With the use of modern molecular techniques, population genetics of parasites can be conducted using several genetic markers including the ITS (the ribosomal internal transcribed spacer) regions and the mitochondrial DNA (mtDNA), especially the nad4 (nicotinamide adenine dinucleotide dehydrogenase subunit 4) genes (Gharamah et al. 2012). As a result, this study analysed the ribosomal internal transcribed spacer gene in order to gain a better understanding of genetic relationship between *H. contortus* populations isolated from the five districts of Limpopo province in comparison to *H. contortus* populations isolated elsewhere in the world.

1.3 Main objective of the study

To determine the GIN species of sheep, genetic deversity of *Haemonchus contortus*, seasonal prevalence and level of use of anthelmintics by resource-poor livestock farmers in the Limpopo province of South Africa

Specific objectives of the study

- To determine seasonal prevalence of gastrointestinal nematodes infections in sheep from five districts of Limpopo province.
- To evaluate the rural resource-poor sheep farmer’s knowledge on anthelmintic use using questionnaire survey.
- To determine the prevalence of anthelmintic resistance to the three most widely used anthelmintics using faecal egg reduction tests and *in vitro* methods.
- To determine population genetic structure of isolates of the most prevalent nematodes species, *H. contortus* using the ITS2 gene.

1.4 Hypotheses

- Gastrointestinal parasite load is higher during the hot wet summer months and lower during the dry cooler months of the year.
- The resource poor farmer in Limpopo province has no adequate knowledge on the correct use of anthelmintics.
- Gastrointestinal nematodes has developed resistance to the three most widely used classes of anthelmintic drugs.

- There is no genetic diversity within and among *Haemonchus contortus* populations from Limpopo province.

1.5 Thesis organization

This thesis is presented in seven stand-alone chapters containing:

Chapter 1: Introduction

Provides background of the concepts of the study including statement of the problem, aim, objectives and hypotheses.

Chapter 2: Literature review

Provides literature on seasonal prevalence of gastrointestinal nematodes, risk factors associated with occurrence of anthelmintic resistance, anthelmintic resistance in gastrointestinal nematodes and population genetic structure of *Haemonchus contortus.*

Chapter 3: Seasonal prevalence of gastrointestinal nematodes infecting sheep in Limpopo province, South Africa

It outlines the introduction, materials and methods, results and discussion of seasonal prevalence of gastrointestinal nematodes (GINs) study in the five districts of Limpopo province.

Chapter 4: Risk factors associated with occurrence of anthelmintic resistance of gastrointestinal nematodes of sheep of resource-poor farmers in Limpopo province, South Africa

It outlines the introduction, materials and methods, results and discussion on the evaluation of knowledge of resource-poor sheep farmers in Limpopo province of South Africa on the use of anthelmintics.

Chapter 5: Anthelmintic resistance in gastrointestinal nematodes of sheep in Limpopo province, South Africa

It outlines the introduction, materials and methods, results and discussion of anthelmintic resistance (AR) study in five districts of Limpopo province using *in vivo* Faecal Egg Count

Reduction Test (FECRT) and *in vitro* Egg Hatch Test and Micro Agar Larval Development Test (MALDT).

Chapter 6: Genetic analysis of isolates of *Haemonchus contortus* from five districts of Limpopo province, South Africa: Implications for spread of anthelmintic resistant isolates

It outlines the introduction, materials and methods, results and discussion of genetic diversity and population genetic structure of *Haemonchus contortus* in the five districts of Limpopo Province, South Africa and the rest of the world.

Chapter 7: Conclusion and recommendations

It outlines conclusions and recommendations drawn from important observations and opinions that reflect serious constraints for resource-poor farmers in Limpopo province of South Africa.

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CHAPTER 2: LITERATURE REVIEW

2.1 Helminths of the gastrointestinal tract of small stock

The helminths of veterinary importance infesting the intestinal tract of small ruminants are numerous and have different areas of predilection within the gastrointestinal tract (Soulsby 1982; Morgan 2013). Morgan (2013) classified the helminths infesting livestock according to these areas of predilection as follows: (1) the helminths of the oesophagus and of the omasum such as *Cotylophoron* spp*, Gongylonema pulchrum,* and *Calicophoron* spp; (2) the helminths of the abomasum such as *Haemonchus contortus, Teladorsagia circumcincta, Teladorsagia trifurcata, Parabonema* spp*.* and *Trichostrongylus axei*; (3) the helminths of the small intestine such as *Avitellina centripunctata, Bunostomum trigonocephalum, Cooperia curticei, Cooperia surnabada, Gaigeria pachyscelis, Moniezia expansa, Nematodirus battus, Nematodirus filicollis, Nematodirus spathiger, Strongyloides papillosus, Trichostrongylus capricola* and *Trichostrongylus vitirinus* and lastly (4) the helmints of the large intestine such as *Chabertia ovina, Oesophagostomum columbianum, Oesophagostomum venulosum, Skjabinema ovis, Trichuris ovis* and *Trichuris skrjabini*. Studies in Kenya reported *Haemonchus, Trichostrongylus, Cooperia* and *Oesophagostomum* as widely encountered strongyle genera of small ruminants (Kanyari et al. 2009).

2.2 Nematodes of small ruminants of economic importance

2.2.1 *Haemonchus contortus*

Haemonchus contortus infestation represents the primary constraint to profitable small stock production in many regions of the world (Li et al. 2016). Haemonchosis caused by this parasite is a predominantly, highly epidemic and economically important disease of sheep and goats (Mortensen et al. 2003). The parasites are blood feeders that cause anaemia and reduced productivity and can lead to death in heavily infected animals (Githigia et al. 2001). The females of *H. contortus* can lay 5000 to 15000 eggs per day in the host animal's faeces (Hansen and Perry 1994) and it has been estimated that each worm sucks about 0.05 ml of blood per day by ingestion or seepage from lesions (Urquhart et al. 2000). An average of 10,000 adult worms is enough to kill a sheep or goat (Burke 2005). The *H. contortus* nematode pierces the lining of the abomasum, causing blood plasma and protein loss in the host and the pathogenic effects of this nematode result from the inability of the host to compensate for the blood loss (Bowman 1995). At peak infection, naturally acquired populations of *H. contortus* may remove one fifth of the circulating erythrocyte volume per day from lambs and may remove an average of one tenth of the circulating erythrocyte volume per day over the course of nonfatal infections lasting two months (Bowman et al. 2003).

2.2.2 *Teladorsagia circumcincta*

Females of *Teladorsagia circumcincta* species are less fertile than *H. contortus*, with an average egg production of 100–200 eggs per female per day (Cole 1986). *Teladorsagia* does not feed on blood, and the main pathogenic effects are caused by its larval stages. Larval development takes place in the gastric glands, leading to nodule formation in the abomasal mucosa and extensive damage to parietal cells, in turn causing a decrease in hydrochloric acid production (McKellar 1993). The severity of the infection depends on other infections occurring at the same time, nutritional state of the host and also its ability to develop an immunogenic response (Stear et al. 2003). Commonly, moderate or subclinical infections occur, causing diarrhoea, poor weight gain, weight loss and reduced wool production (Zajac 2006).

2.2.3 *Trichostrongylus* species

Infections with *Trichostrongylus* spp. are often difficult to distinguish from malnutrition in the case of low-intensity infections but, if worms are present in high numbers, they may cause protracted watery diarrhoea, which stains the fleece of the hindquarters (black scours) (Taylor 2007). *Trichostrongylus axei,* which lives in the abomasum, is less common and occurs usually in smaller numbers (Donald et al. 1978).

2.3 Life cycle of gastrointestinal nematodes

The life cycles of gastrointestinal nematodes are direct, requiring no intermediate hosts, which applies to all the economically important strongylid parasites of small ruminants (Hansen & Perry, 1994; Urquhart et al. 1996). Adult females in the gastrointestinal (GI) tract lay eggs that are passed out with the faeces of sheep (Figure 2.1). Development occurs within the faeces, then the eggs embryonate and hatch into first-stage larvae (L_1), which moult into second-stage larvae (L_2), shedding their protective sheeth in the process. During this time the larvae feeds on bacteria. The L_2 moult into third-stage larvae (L_3) but retain the cuticle from the previous moult. The L_3 is the infective stage, and these move onto surrounding foliage where they become available for ingestion by grazing small ruminants.

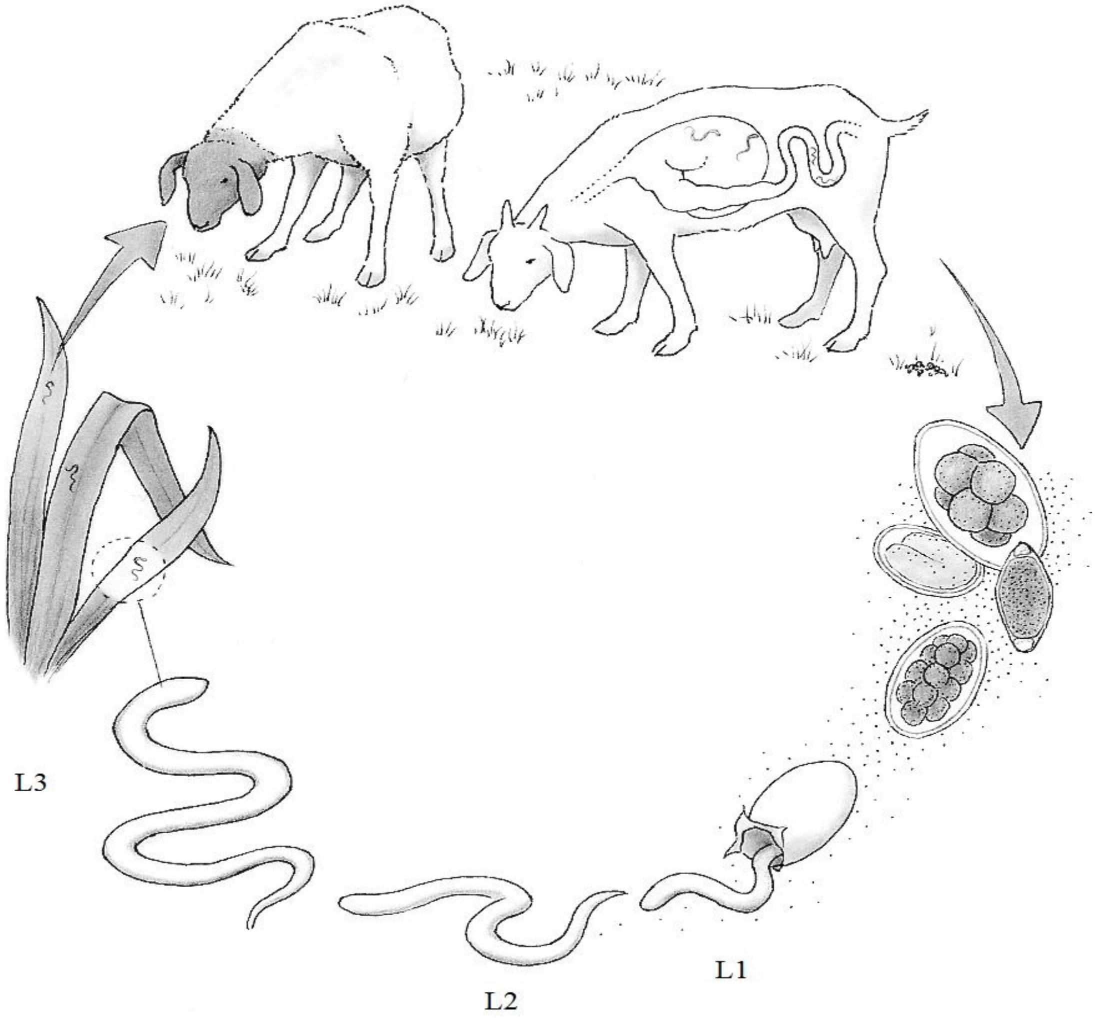

Figure 2.1: Principal life-cycle of gastro intestinal nematodes (Mekonnen 2007)

Immediately after ingestion, the L_3 larvae passes to the abomasum, where they ex-sheathe. The L_3 of the trichostrongyle worms penetrate the epithelial layer of the mucus membrane (in the case of *Haemonchus* and *Trichostrongylus*) or enter the gastric glands in the case of *Teladorsagia*. Under normal circumstances, the L_3 moult within 2–3 days to become fourth-stage larvae (L_4), which remain in the mucous membrane for a further 10 to 14 days. Eventually, the L_4 emerge and moult to become young adult worms. The time between ingestion of L_3 and the parasite becoming mature adults (referred to as the prepatent period) varies between parasite species, but in most cases is between 3 and 5 weeks. *Nematodirus*, *Trichuris*, *Bunostomum*, *Gaigeria* and *Strongyloides* species are exceptions to the lifecycle described above (Soulsby, 1982; Urquhart et al. 1996).

2.4 Treatment and control

Worm control globally is exclusively based on anthelmintic treatments rather than on management procedures of integrated strategies. Many South African small stock farmers depend heavily on the use of anthelmintics to control gastrointestinal nematodes and this has resulted in selection of worm populations that are resistant to anthelmintics (Vatta et al. 2001). The currently available anthelmintics belong to different drug classes i.e. macrocyclic lactones (ML's), benzimidazoles (BZ's), tedrahydropyrimidines-imidazothiazoles, amino-acetonitriles-derivates and spiroindoles (Traversa and Samson-Himmelstjerna 2015). Similarly, the primary means of controlling nematode infections in livestock employed by South African farmers is the use of anthelmintic drugs primarily ivermectin, albendazole and levamisole and although farmers are doing their best to combat nematode infestations, the severity of AR has led to a decrease in their efficacy (Tsotetsi et al. 2013). This justifies an urgent need to find alternatives to synthetic drugs (Shen et al. 2010).

2.5 Modes of action for different anthelmintic classes

Each class of anthelmintics has a distinct mode of action against parasites (Kohler 2001). Imidazothiazoles, such as levamisole, are acetylcholine agonists that targets the nervous system of the parasite (Kohler 2001). These drugs cause muscle contraction and paralysis in the helminth, resulting in the eventual expulsion of the parasite from the body (Craig 1993; Mansour 2002). Macrocyclic lactones act on glutamate-gated chloride channels (GluCl). These drugs cause paralysis of the parasite neuromusculature, including the pharynx, preventing the worm from feeding (Kohler 2001). The target of benzimidazoles is the tubulin within the parasite intestinal cells, which forms into microtubules that are necessary for nutrient acquisition (Sangster and Dobson 2002). Benzimidazoles bind to the β-tubulin component preventing it from forming microtubules within the intestinal cells of the helminth. This impairs the uptake of nutrients and inhibits the transportation of necessary digestive enzymes resulting in parasite death due to starvation (Kohler 2001; Mansour, 2002). Additional effects of benzimidazoles on nematodes include depletion of energy reserves and the inhibition of waste excretion (Vercruysse and Claerebout 2014). The only available amino-acetonitrile derivative on the market today is monepantel (Vercruysse and Claerebout 2014). It acts as an agonist of the mptl-1 channel, a channel belonging to a class of nicotinic acetylcholine receptors. It causes constant fluctuation in muscle ions leading to muscle depolarization and irreversible nematode paralysis (Vercruysse and Claerebout 2014). Benzimidazoles and macrocyclic lactones are effective against the adult and immature stages of the parasite, while the imidazothiazoles are effective against the adults and the later stages of immature larvae (Kohler 2001). The ability of the drug to enter the worm and interact with its target receptor in order to trigger a harmful physiological effect (shown at top for a drug- susceptible worm) is diminished through four principal mechanisms. These mechanisms apply to varying degrees to the major anthelmintic drug classes, as indicted by the relative font of the drug class names at the base of the figure 2.2.

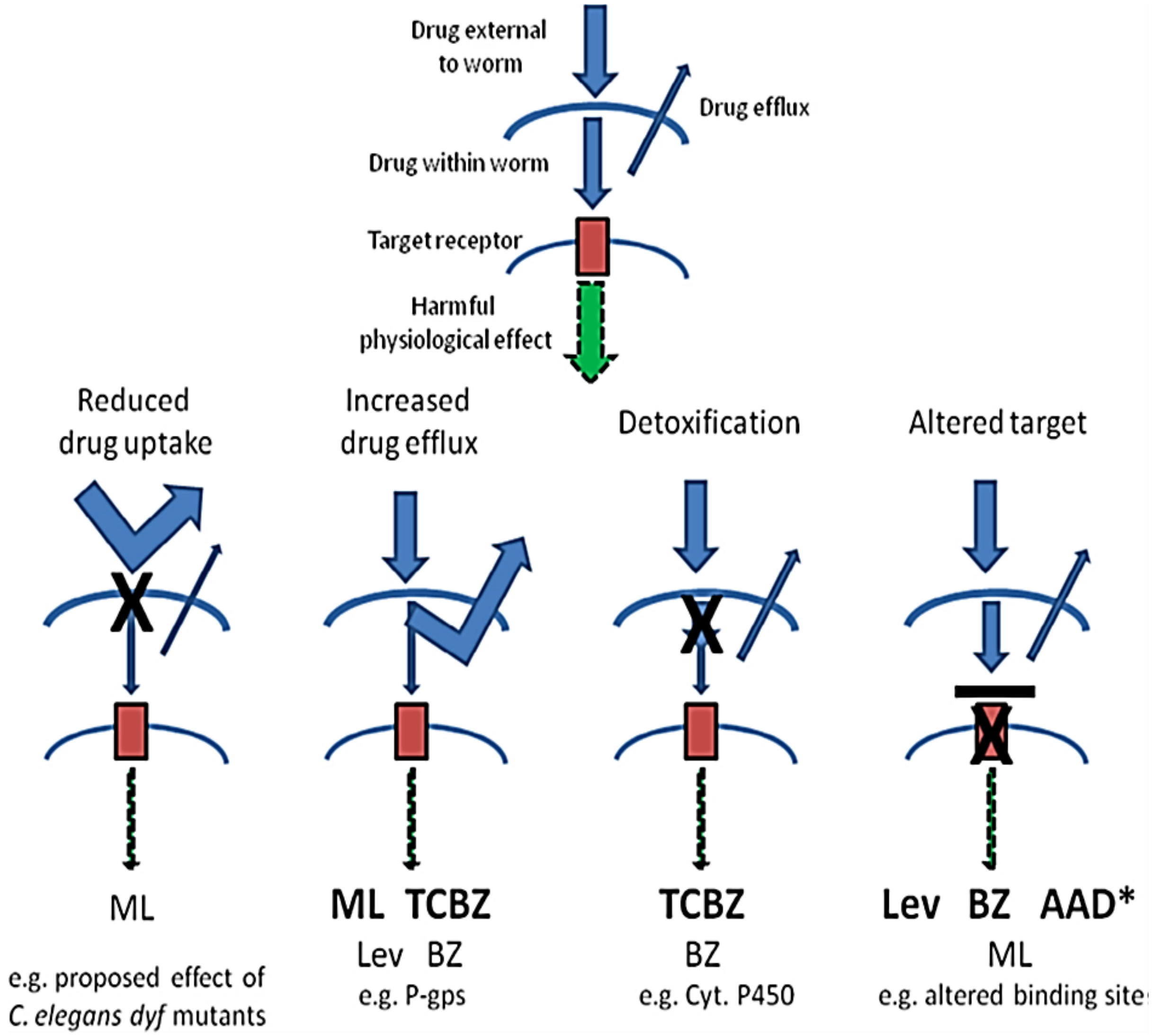

Figure 2.2: Schematic representation of principal anthelmintic resistance pathways, and their relevance to each of the current anthelmintic drug classes. ; ML = macrocyclic lactones, TCBZ = triclabendazole, Lev = levamisole (as a representative of the nicotinic agonist drug class), BZ = benzimidazoles, AAD = amino-acetonitrile derivatives; * denotes

that resistance to the AADs is only characterised in laboratory-selected isolates (Adapted from Kotze et al. 2014)

2.6 Alternative parasite control

Prophylactic strategies such as grazing management, biological control with nematophagous fungi or food supplementation, with leguminous plants accumulating high amounts of condensed tannins that help to control gastrointestinal parasites and also disorders such as bloat, are promising (Barrau et al. 2005). Anthelmintics derived from plant parts that are used traditionally for treatment of parasitic infections in humans and animals may offer an alternative in minimizing some of these problems (Akhtar *et al.,* 2000). In addition, the growth of organic livestock farming globally, which is claimed to be less toxic to human beings and the environment, might favour the continuous use of traditional medicinal plants in Africa for treatment of endoparasitic infections caused by intestinal worms (Nchu et al. 2011).

2.6.1 Copper oxide wire particles (COWP)

Copper is a necessary trace element in the diet of ruminants to facilitate maximum immune response (Salt Institute 2002). Copper Oxide Wire Particles (COWP) has been used for many years to treat copper deficiency (Suttle 1981; Judson et al. 1982, 1984; Langlands et al. 1993; Dewey 1997). But COWP are not only an efficient and effective means of treating copper deficiency in grazing livestock, they can also be potentially useful as an anthelmintic (Dewey 1997). After dosing, COWP flow together with ingesta from the rumen and lodge in the folds of the sheep’s abomasum where the low pH induces the release of high concentrations of soluble copper, which have an adverse effect on abomasal species of nematodes (Knox 2002). Because of the rapid increase in AR, this control method is continually being evaluated. The reported anthelmintic effect of COWP has been seen in numerous studies (Bang et al. 1990a; Chartier et al. 2000; Nyman 2000; Knox 2002).

Use of COWP should be combined with other worm control strategies. Selective treatment is advised to minimize development of nematode resistance to available anthelmintics and/or COWP. Selective treatment can be implemented using the FAMACHA© system. Only animals with anaemic FAMACHA© scores should be treated. Other control methods include rotational grazing, avoidance of over-grazing, mixed species grazing, use of resistant breeds or resistant animals within a breed, good nutrition, feeding with condensed tannin-rich plants such as *Sericea lespedeza*, and eliminating wet spots in pastures where barber pole worm flourishes (Burke and Miller 2006). Professional consultation from veterinarians and extension agents is strongly advised to assess farm conditions, feeding programmes, and other management and environmental factors that will affect copper oxide metabolism (Burke and Miller 2006).

2.6.2 Pasture rotation and nutrient supplementation

Rotational grazing is allowing forages in some pastures to rest and regrow while grazing another pasture (Kim 2004). Proper pasture rotation allows time for on-pasture larvae to die out before they can be re-consumed and for grasses to grow higher than the larvae can climb (Machen et al. 1998). Since in most developing countries, the system of grazing that is preferred is communal grazing, rotational grazing between sheep and cattle should be considered a practical approach to reducing contamination of pastures with parasites (Mafisa 1993). Githigia et al. (2001) further indicated the necessity of moving weaned lambs to a clean pasture before the expected mid-summer rise in parasitic infection. Disadvantages of rotational grazing system include significant initial investment cost and increased management (Ball et al. 1999).

2.6.3 Improved nutrition

Protein and herb supplements improve the health of the digestive tract, lessening the effects of infection and increasing host resilience (Houtert and Sykes 1996; Williams 2010). Considerable attention has been directed towards demonstrating the deleterious impact of intestinal parasites on nitrogen utilization and livestock performance, but the reciprocal relationship, that well-nourished animals resist intestinal parasitism better than those less adequately fed, is gaining increasing prominence as researchers recognize that prolonged parasitism in protein deficient animals can be reversed by protein supplementation (Coop and Holmes 1996). Studies in lambs have demonstrated that high-protein diets enhance the development of immunity against *H. contortus* (Perez et al. 2001), *T. colubriformis* (Kambara et al. 1993), and *O. circumcincta* (Coop et al. 1995). Malnutrition can reduce the success of drug interventions and the reduced efficacy is likely to promote selection of drug-resistant parasites further limiting chemotherapeutic control of GI nematode infections and for this reason nutritional intervention should precede drug treatments to ensure maximal drug effectiveness during intervention programs (Koski and Scott 2001).

2.6.4 Targeted drenching

Targeted drenching is based on the selective treatment of individual animals that are diagnosed as being infected and presenting clinical symptoms of the disease (Mahieu et al. 2007). The chemical control measures include the targeted, selective use of anthelmintics, which is achieved either by monitoring and treating individual animals, or by targeting specific nematode species and consequently minimising the use of anthelmintics (Athanasiandou et al. 2008).

The FAMACHA© system was developed by South African scientists and veterinarians. This is a method of targeted treatment and is a strategy for conserving the efficacy of existing drugs (Malan et al. 2001). The system is based on the fact that sheep and goats suffering from haemonchosis show varying degrees of anaemia, which can be evaluated clinically by examination of the ocular mucous membranes. With the help of a colour chart, animals are scored in one of five colour categories (from red, non-anaemic, to very pale, severely anaemic). Only those animals in need of treatment are treated. It is a low-cost tool that may greatly influence management practices in small ruminants. The system was developed in South Africa and uses the evaluation of anaemia, based on clinical evaluation of the colour of the lower eyelid mucous membrane, as a morbidity marker for haemonchosis (Malan et al. 2001; Vatta et al. 2002; Van Wyk and Bath 2002). The system has been tested in different production systems and countries, where *Haemonchus contortus* is the major gastrointestinal nematode helminth of sheep (Di Loria et al. 2009). The studies have shown that, by using the system, animals in greater need of anthelmintic treatment can be identified and treated selectively. This way, it might be possible to reduce numbers of anthelmintic treatments in a flock/herd and to maintain a helminth population in refugia in animals that are not deemed to require treatment (Malan et al. 2001; Vatta et al. 2002; Kaplan et al. 2004). Nevertheless, possible variations in the results obtained by the system can occur among management systems, animal breeds, types and ages of animals, system operators, environments and facilities (Moors and Gauly 2009; Reynecke et al. 2011a, b) and must be studied before the system can be applied in a given situation.

This practice can potentially prevent the overuse of anthelmintics and consequently minimize chances of parasite resistance to anthelmintics (Van Wyk and Bath 2002). FAMACHA$^{©}$ also provides producers with a tool for genetic selection because producers will be able to identify animals with high resistance and resilience, which seem to be inherited traits in small ruminants (Leite – Browning 2006). FAMACHA$^{©}$ system can also be used to select replacement animals that are resistant and/or resilient to *H. contortus* through proper record keeping. Animals that require fewer deworming treatments should be retained, while those that require more frequent treatments should be culled or removed from the flock (Burke and Miller 2006).

In the summer-rainfall area of South Africa, *Haemonchus* infection is seasonal. Following the dry winter period (June–August), a spring rise in FEC occurs due to both a resumption of transmission and the development of hypobiotic worms into egg-laying adults (Barth et al. 2001). Transmission of the parasite on pastures is slow during the spring, but as rainfall, temperatures and vegetative ground cover increase (conditions favourable for *Haemonchus* spp.) towards mid-summer (December), transmission of the parasite also occurs with increasing frequency. Parasite burdens tend to reach maximum levels in the late summer and early autumn. In line with this seasonal trend, FAMACHA$^{©}$ examinations are carried out less frequently (e.g. every 3 weeks) during the spring and early summer, rising after good rains to weekly during the usually short peak of worm infestation. At the start of the worm season sheep must be treated when scored as 4 or 5. Sheep scored as 3 which is considered to be borderline, should however be treated when potential outbreaks of clinical *haemonchosis* are expected (Kaplan et al. 2004).

A great advantage of the system is that it can be easily understood and learnt by poorly literate farmers. This has been demonstrated in commercial farms, where the system has found great acceptance, and in resource-poor farming systems (Vatta et al. 2002). Most of the reports of anthelmintic resistance are from large scale commercial or institutional farms. Under these conditions, the selection pressure for anthelmintic resistance is often intense with, for example, frequent anthelmintic treatment of the whole herd. This in itself exposes a greater proportion of the nematode population to anthelmintics and leaves fewer worms *in refugia* than would be the case, for example, if only those individual animals showing signs of helminthosis were drenched (Vatta and Lindberg 2006). The frequent use of anthelmintics increases the frequency with which individual nematodes and their offspring are exposed to anthelmintics as well as the probability that a nematode will be exposed to an anthelmintic within a certain period of time. Large herd size has been reported as a risk factor for the occurrence of resistance (Wanyangu et al. 1996).

2.6.5 Selecting for nematode/*Haemonchus* resistant and resilient animals

Nematode resistance includes the initiation and maintenance of a host response that prevents, reduces, or clears parasitic infection (Hooda et al. 1999; Bricarello et al. 2004). Resistant animals are not completely immune to the infection, but they have a lower parasitic load than susceptible animals, as measured by fewer eggs in their feaces. This resistance is based on the immunological capabilities of each individual when challenged with parasites (Gill 1991). Resilience is the capacity of an animal to compensate for the negative effects of parasitism by the maintenance of productive parameters. Sheep in general show simultaneously high resistance and resilience to haemonchosis. Some breeds have moderate or low resistance with relatively high resilience, allowing them to have productivity similar to those that are naturally resistant (Alba-Hurtado et al. 2010).

Identification and selective breeding of animals with higher genetic resistance to gastrointestinal nematodes is an attractive alternative (Raadsma and Tammen 2005). The use of genetically resistant animals may also optimize the efficacy of anthelmintic use by delaying the development of parasite resistant populations and extending the useful life of an anthelmintic. Several sheep breeds have shown a natural resistance to gastrointestinal nematodes, such that many are currently being studied to develop their selective breeding and potential commercial production traits (Gamble and Zodiac 1992; Amarante et al. 2004; Mugambi et al. 2005).

2.6.6 Ethnoveterinary medicine

In traditional livestock systems, animals are affected by diseases, such as gastrointestinal nematode parasitism which is highly prevalent and leads to a huge economic loss. This loss results from both the mortality of young animals and the decrease in production (Krecek and Waller 2006).

Developed and developing countries show a great interest in indigenous medicine as their primary method of animal health care, since it is inexpensive and readily available for usage (McGaw et al 2000). Traditional South African medicine makes use of a variety of plants in the treatment of many livestock diseases in Kwazulu-Natal (Cunningham and Zondi, 1991), the Eastern Cape (Matika et al. 2000) and North-West Province (Van der Merwe et al 2001). Ethnoveterinary investigations have been undertaken for the Mopani and Vhembe districts in Limpopo Province (Luseba and Tshisikawe 2010).

Since plant derivatives continue to serve as important sources of drugs and several plant-based drugs are extensively used, there is a need for the development of sustainable strategies to exploit Africa's rich plant biodiversity. There is also an urgent necessity to characterize the ethnoveterinary properties of increasingly endangered plant species in order to improve animal production on the continent (Okoli et al. 2010).

2.6.7 Biological control

Nematophagous fungi present themselves as natural enemies of gastrointestinal helminth parasites. They can be found in diverse environments and earlier and more recent studies have shown that they are effective as bio-control agents (Kerry 2000; Araujo et al. 2013). Out of the many natural antagonists of helminths, including protozoa, bacteria, viruses, mites, beetles and fungi that have already been described as biological controllers (Gronvold et al. 1996), nematophagous fungi are showing the most potential as biocontrol agents (Ward et al. 2012).

2.7 Population genetics and phylogenetic analysis

Control of parasitic nematodes in livestock focuses on the use of anthelmintic drugs and farm management (Gasser et al. 2008). However, the knowledge derived from genetic information regarding the parasite such as genetic diversity and genetic relationships through phylogenetic analysis is necessary to increase understanding of the ecology, epidemiology and evolution of parasitic nematodes and also to increase the efficacy of control programs (Jacquiet et al. 1995). Phylogeny studies the hierarchical relationships between genes from different species and relationships between genes from individuals within a species which are not necessarily hierarchical (Posada and Crandall 2001).

According to Felsenstein, (2003), phylogenetic relationships, are patterns of shared common evolutionary history between biological replicators, such as species or genes. Phylogenetic analysis using DNA or protein sequences has been used for many years to investigate the evolutionary history of unicellular to multicellular organisms (Nei and Kumar 2000). Most of the previous phylogenies of kinetoplastids were based on analysis of variation in SSU rRNA genes (Hamilton et al. 2007). Molecular phylogenetics is the study of evolutionary relationships among taxa or genes and is a combination of molecular and statistical techniques (Felsenstein 2003). In general, organisms with more similar genes are more closely related, i.e. they are likely to share a more recent common ancestor than are distantly related taxa (Salemi and Vandamme 2003).

The relationships between species can be represented by a phylogenetic tree, which is a graphical representation with nodes and branches in a bi- or multifurcating structure. Methods to construct phylogenetic relationships can be classified into two groups, i.e. cladistic methods (maximum parsimony, maximum likelihood and bayesian) and phenetic methods (distance matrix methods such as neighbor joining) (Posada and Crandall 2001).

Haemonchus contortus is one of the most economically significant livestock parasites worldwide and is an important experimental model for the strongylid nematode group that includes many important human and animal pathogens (Knox et al. 2003; Gilleard 2006). It is one of the more amenable parasitic nematodes to genetic analysis, having high levels of genetic polymorphism both within and between isolates and being one of the very few parasitic nematode species in which genetic crosses between isolates have been successfully undertaken (Troell et al. 2006).

Extremely high levels of genetic diversity have been reported in many species of nematodes (Gilleard and Beech 2007). In the case of *H. contortus*, this high genetic diversity is predicted to be a result of a large population size, rapid rate of reproduction and wide geographical spread (Prichard 2001). The naturally large population size of *H. contortus* is thought to be further increased by host movement. Acoordingly, Blouin et al. (1995) found that variations in mitochondrial DNA sequence in *H. contortus* populations across four North American states were higher within populations than between populations. With the limited range of dispersal of the free-living stages, this was predicted to be a result of extensive movement of sheep between farms/states. The implication of this kind of extensive movement is that, the spread of resistance alleles is wider. Population genetic studies of *H. contortus* have been conducted in a wide range of geographical regions of the world, including Australia, Brazil, Europe, Malaysia, and the USA (Troell 2006; Hunt eat al. 2008). In China, a study conducted by Yin et al. (2013), nucleotide sequence analyses revealed 18 genotypes (ITS2) and 142 haplotypes (nad4) among the 152 worms, with nucleotide diversities of 2.6% and 0.027, respectively, consistent with previous reports from other countries, including Australia, Brazil, Germany, Italy, Malaysia, Sweden, the USA and Yemen. Population genetic analyses revealed that 92.4% of nucleotide variation was partitioned within populations; there was no genetic differentiation but a high gene flow among Chinese populations; some degree of genetic differentiation was inferred between some specimens from China and those from other countries (Yin et al. 2013). In general, the results from the first Chinese study of genetic variation within *H. contortus* revealed high within-population variation, low population genetic differentiation and high gene flow of *H. contortus*. The results also indicated that on a global scale, there is no or a low level of genetic differentiation among *H. contortus* populations from the same continent, and significantly higher levels of genetic differentiation among those from different continents.

Population genetic studies of *H. contortus* have shown that this species exhibits high within population variation and low genetic differentiation within continuous geographical regions, likely ascribing to high gene flow influenced by host movement (Boulin et al. 1995). However, strong barriers to gene flow have been observed on a global scale, which appear to be attributed to poor dispersal ability of the parasite and restricted opportunities for host movement across continents (Troell et al. 2006). A study by Chaudhry et al. (2015) concluded that there was very little difference in overall genetic diversity between the 12 populations of *H. contortus* in across Southern India.

Information on the genetic diversity and population structure is important in understanding the genetic attributes influencing pathogenicity and response to treatment plan of any disease pathogen, as well as in tracing of the accelerating spread of drug resistance among parasitic nematodes (Kaplan 2004). Mitochondrial DNA (mtDNA) sequences have been useful markers in studies of genetic diversity and population structure in livestock (Muchadeyi et al. 2008) and animal pathogens (Walker et al. 2007). Mitochondrial genomes evolve 5–10 times faster than nuclear genomes (Brown et al. 1982) most probably due to lack of replication repair mechanism (Clayton 1982). It is this trait that makes them suitable for discriminating closely related organisms (Kaplan 2004) especially at the species and sub-species levels (Galtier et al. 2009). Networks analysis of mtDNA sequences has been widely used in phylogenetic analysis (Bandelt et al. 1999).

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CHAPTER 3: SEASONAL PREVALENCE OF GASTROINTESTINAL NEMATODES INFECTING SHEEP IN LIMPOPO PROVINCE, SOUTH AFRICA

Abstract

This study determined seasonal prevalence of gastrointestinal nematodes in five districts (Capricorn, Mopani, Sekhukhune, Vhembe, and Waterberg) of Limpopo province. Faecal samples were collected from sheep on a bi-monthly basis from 156 sheep in each district (total = 780 sheep) for a period of 21 months. Samples were analysed with McMaster technique and revealed high prevalence of infection ranging from 88 to 99% with mean faecal egg counts ranging between 1210 and 1861 eggs per gram (EPG) and 75 – 83% with mean egg counts ranging between 453 – 1202 EPG during hot wet season and cold dry season respectively in all districts. There was no significant difference in both nematode prevalence and mean egg counts ($p > 0.05$) between the hot wet and cold dry seasons. *Haemonchus contortus* was the most dominant nematode species (70 – 93%) in all districts followed by *Trichostrongylus/Teladorsagia* spp. (5 – 28%) and *Oesophagostomum columbianum* (<5%). FAMACHA$^{©}$ results revealed a mean score of 3 for all the districts except Vhembe, with a score of 4 during the hot wet season and a score of 2 for all districts during the cold dry season except Waterberg which had a score of 1. There was a positive correlation between mean minimum temperature and mean FEC ($r = 0.482$; $p = 0.01$) and between rainfall and FEC ($r = 0.755$; $p = \leq 0.01$) in all districts. Furthermore, there was strong correlation ($r = 0.959$; $p = \leq 0.01$) between faecal egg count and clinical anaemia in sheep. FAMACHA$^{©}$ scores suggests that *Haemonchus contortus* in particular was the main contributory factor to anaemia.

Keywords FAMACHA$^{©}$; Eggs per gram; Gastrointestinal nematodes; sheep; Seasonal prevalence; Limpopo province; South Africa

3.1 Introduction

Gastrointestinal nematodes (GINs) are responsible for substantial losses of production in the animal production industry. In small stock, GINs can result in anaemia due to the blood-sucking activities of nematodes such as *Haemonchus* spp which impacts negatively on the profitability of the farm (Raharivololona and Ganzhorn 2010). The annual costs associated with parasitic diseases in small ruminants are estimated to be tens of billions of US dollars worldwide, according to the sales of anthelmintic drugs by pharmaceutical companies, excluding production losses (Sackett and Holmes 2006). Seasonal variations also play a vital role in transmission of GINs and as such, changes in humidity and temperature can influence development, survival and transmission of parasites in the environment (Raharivololona and Ganzhorn 2010).

Gastrointestinal parasites multiply more rapidly during the warm, rainy season and as a result, months that form summer season have a conducive environment for gastrointestinal parasites development (Aga et al. 2013). Factors such as humidity and vegetation cover also have an effect on patterns of parasite development (Sun et al. 2018). In general, the combined effects of these factors are responsible for the seasonal fluctuations in the availability of L_3 on pasture, and subsequently in the prevalence of worm burdens in the hosts. The seasonal variation of parasite population dynamics has been recounted in several research initiatives in many African countries including South Africa (Van Wyk 1985); Nigeria (Fakae 1990); Zimbabwe (Pandey et al. 1994); Kenya (Nginyi et al. 2001) and Ethiopia (Debela 2002). The ideal temperature range for larval development of many nematode species in the microclimate of the pasture lies between 22 and 26°C while the optimal humidity is close to 100%. Desiccation from lack of rainfall kills eggs and larvae rapidly and is the most lethal of all climatic factors (Getachew et al. 2007). Wet seasons on the other hand favour the vigorous growth of pasture which in turn attracts grazing sheep and goats leading, not only to further contamination of pasture, but also providing an opportunity for the existing infective larvae to encounter their preferred hosts (Getachew et al. 2007).

The most important strongylid nematodes of sheep and goats in sub-Saharan African countries are: *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus spp.* (*T. axei, T. colubriformis* and *T. vitrinus*). Other species of lesser importance include *Pseudomarshallagia* (*Longistrongylus*) *elongata, Nematodirus spp.* (*N. spathiger* and *N. filicollis*), *Cooperia curticei, Bunostomum trigonocephalum, Gaigeria pachycelis,* *Oesophagostomum spp.* (*O. venulosum* and *O. columbianum*) and *Chabertia ovina* (Hansen and Perry 1994). According to Charlier et al. (2014) and Rinaldi and Cringoli (2014), *Haemonchus, Ostertagia, Teladorsagia, Trichostrongylus, Cooperia, Oesophagostomum* and *Chabertia* genera are on top of the list of nematodes that continue to cause significant economic burden to the global animal production industry.

In South Africa, species from the genus *Haemonchus* are the most important helminth parasites of small ruminants in the summer rainfall areas of the country, both for the commercial (Van Wyk 2001) and resource poor farmers (Tsotetsi and Mbati 2003). It poses a threat from the month of November to April when ambient temperature and rainfall are ideal to the parasite to complete its life cycle on pasture. However, *H. contortus* may also be a threat in the cooler months of the year and can occasionally become problematic in the semi-arid regions of the country during wet years, as well as in the winter and non-seasonal rainfall areas (Vatta and Lindberg 2006). The *Ostertagia* sp. infection has a characteristic watery diarrhoea which usually persists coupled with periods of constipation. Lesions can be readily seen in the abomasum and small brown purple spots due to bleeding may be visible where the worms have been feeding (Myers and Taylor 1989). Characteristic lesions of *Ostertagia* sp. infection are small, umbilicated nodules 1 – 2 mm in diameter (Myers and Taylor 1989). On the other hand, *Teladorsagia* does not feed on blood, and the main pathogenic effects are caused by its larvae which develop in the gastric glands, leading to nodule formation in the abomasal mucosa and extensive damage to parietal cells (McKellar 1993). Infections with *Trichostrongylus* are often difficult to differentiate from malnutrition in the case of low-intensity infections (Taylor et al. 2007) but a high worm burden causes protracted watery diarrhoea, which stains the fleece of the hindquarters (black scours) (Roeber et al. 2013). *Cooperia curticei* and *N. spathiger* are also common parasites of the small and/or large intestine, whilst *C. ovina* is less common (Zajac 2006). Individually, these species have relatively low pathogenicity, but may cause parasitic gastroenteritis in grazing small stock (Taylor 1986).

It is of utmost importance to monitor the prevalence and distribution of livestock helminth species in order to plan sustainable control using targeted treatment and/or targeted selective treatment strategies (Cringoli et al. 2008; Kenyon et al. 2009), hence the objective of this study was to determine seasonal prevalence of gastrointestinal nematodes infections in sheep from five districts of Limpopo province.

3.2 Materials and methods

3.2.1 Study area

The study was conducted on randomly selected small-scale farming locations in five districts of Limpopo Province: Capricorn, Sekhukhune, Waterberg, Mopani and Vhembe (Figure 3.1) of which the participaning farmers were randomly derived from the extension officers and animal health technician’s data base in the districts.

3.2.2 Meteorological data

Meteorological data such as temperature, rainfall and humidity for the selected study sites was obtained from South African Weather Service (SAWS) starting from January 2017 to November 2018.

Ethical clearance

The study was approved by the scientific committee of Integrated Pest Management, North-West University, with reference no: NWU-01252-19-A9. Permission to collect study samples was granted by participating resource poor-farmers.

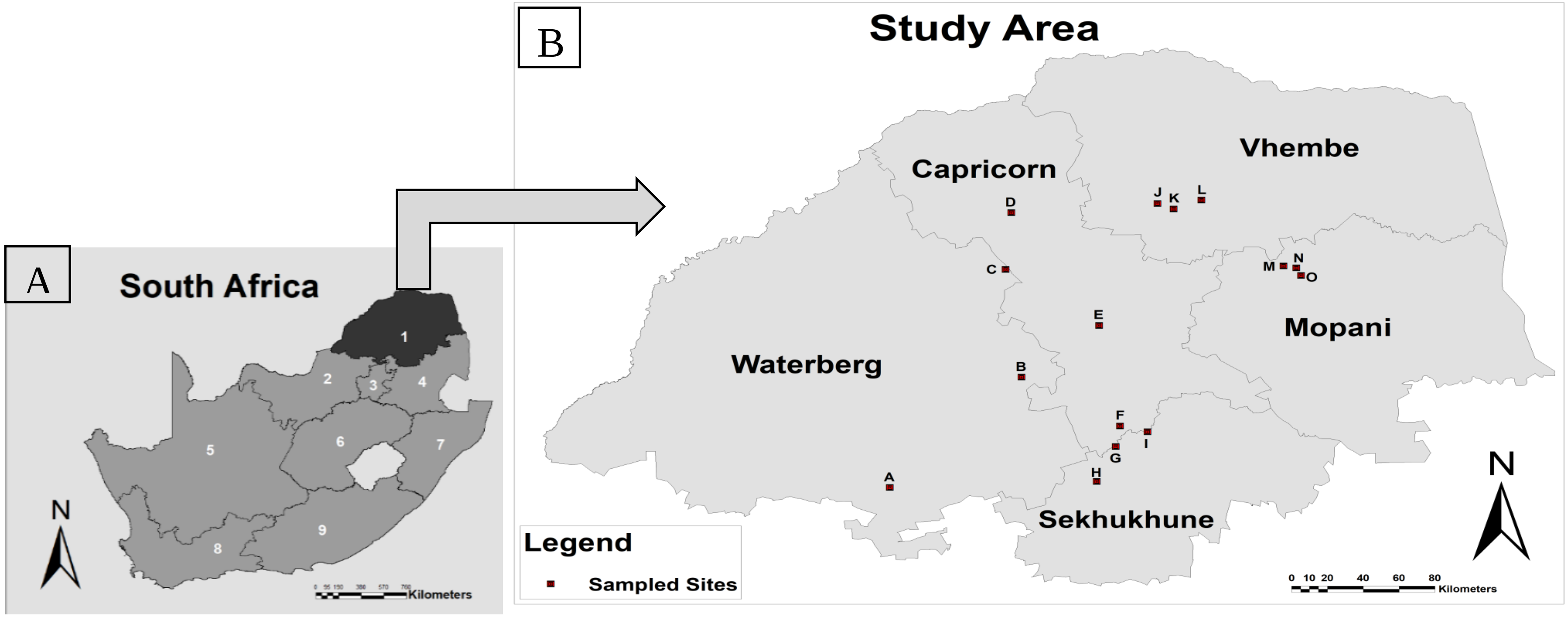

Figure 3.1: Map showing study areas. (A) Map showing nine provinces of South Africa with Limpopo province shown in a dark grey colour. (B) Locality map depicting study sites from Limpopo districts for seasonal prevalence of gastrointestinal nematodes: (a) Bela-Bela (b) Mokopane and (c) Ga-Ramela village from Waterberg district; (d) Ga-Kobe village, (e) Makotse village and (f) Tooseng village from Capricorn district; (g) Malope village, (h)Tompi Seleka college and (I) Strydkraal village from Sekhukhune district; (J) Madodonga village, (k) Ha-Ramantsha village and (l) Louis Trichardt from Vhembe district; (m) Ga-Maupa village, (n) Mamokgadi village and (o) Mamatlepa village from Mopani district

3.2.3 Sample collection

3.2.3.1 Faecal sample collection

Faecal samples were collected between March 2017 and November 2018 on a bi-monthly basis from 780 sheep with n = 156 in each district. The animals were ear tagged so that samples could be taken from the same animals throughout the duration of the study period. Faecal samples were collected directly from the rectum of sheep (Plate 3.1) and stored in a cooler box. They were transported immediately to the Helminthology Laboratory of the Agricultural Research Council, Onderstepoort Veterinary Institute for analysis.

Plate 3.1: Collection of faecal samples from the rectum of sheep

Individual nematode egg counts for each sheep were determined by a modified McMaster technique with detection limit of 100 eggs per gram (EPG) (Reinecke, 1983).

3.2.3.2 FAMACHA© scores

FAMACHA© eye-colour score estimations (Bath et al. 2001; Van Wyk and Bath 2002) were also performed for each study animal. The colour of the mucous membrane of the lower eyelid of each animal was examined in good natural light (Plate 3.2) and compared to the colours of the FAMACHA© chart (Bath et al. 2001; Van Wyk and Bath 2002).

Plate 3.2: Assessing anaemia using a Faffa Malan Chart (FAMACHA©) system

Each sheep was scored on a scale of 1–5 (1 = red, non-anaemic; 2 = red-pink, non-anaemic; 3 = pink, mildly anaemic; 4 = pink-white, anaemic; 5 = white, severely anaemic).

3.2.3.3 Microscopic determination

The McMaster technique (Reinecke 1983) was used for nematode egg count. Briefly, 2 grams of faeces were weighed, and 58 mL of 40% sugar solution added to the sheep faeces as a floatation medium. Samples were thoroughly crushed and dissolved using a blender. Two chambers of the McMaster slide were filled with suspension of faeces in flotation fluid using a pasture pipette and the slides were allowed to stand for about 4 minutes so that the eggs could float on the surface of the floatation medium and lie in contact with the upper glass of the chamber. A dissecting microscope (Nikon Eclipse E100 Company, Japan) was used for egg detection at x 40 magnification; egg per gram counts of faeces were performed by counting all the eggs in the two chambers of the McMaster slides and egg counts were determined by adding the total number of eggs in the two chambers and multiplied by 100.

3.2.3.4 Meteorological data

Meteorological data from the collection sites starting from January 2017 to November 2018 was obtained from SAWS. The data collected included temperature, rainfall and humidity.

3.2.3.5 Faecal culture and larval identification

Larval cultures were prepared according to the method described by Reinecke (1983). Faecal pellets were broken down in a large plastic tray and mixed with a similar volume of vermiculite, to improve the aeration of the culture and to facilitate the maximum hatching of the eggs. A 2 cm thick wooden stick was placed upright in the centre of a 1l glass jar, the faeces were slowly added to the jar and gently compacted with the second stick until the layer of faeces was 5 to 7 cm thick. The stick was removed at this point. A hole remained at the centre of the jar and was used for aeration. The outside of the jar was thoroughly cleaned to avoid contamination.

By using a water bottle a limited amount of water was sprayed onto the inside of the jar and on the compacted faecal mixture without soaking it in order to introduce some moisture, which was necessary for the eggs to hatch. The lid was then lightly screwed on to the bottle and incubated at 27 °C for 7 days. Plate 3.3 shows larval cultures in the incubator at day 7.

Plate 3.3: Larval cultures in the incubator 7 days post culture ready for harvest of L_3 larvae

The culture was harvested by rinsing the sides of the culture jar into a 100ml glass bottle, while holding the bottle at a slant position. Sedimented fluid containing nematodes larvae was transfered into a petri dish. Using a Pasteur pipette, a small droplet of the sedimented fluid from the petri dish was transfered onto a microscope slide. Thereafter a drop of iodine was added, and a coverslip gently placed over the drop. The proportion of nematode species was determined by identifying the first 100 larvae per culture and expressing it as a percentage. The larvae were identified using a light microscope at 10x using the key of Van Wyk and Mayhew (2013) as shown in figure 3.2 below.

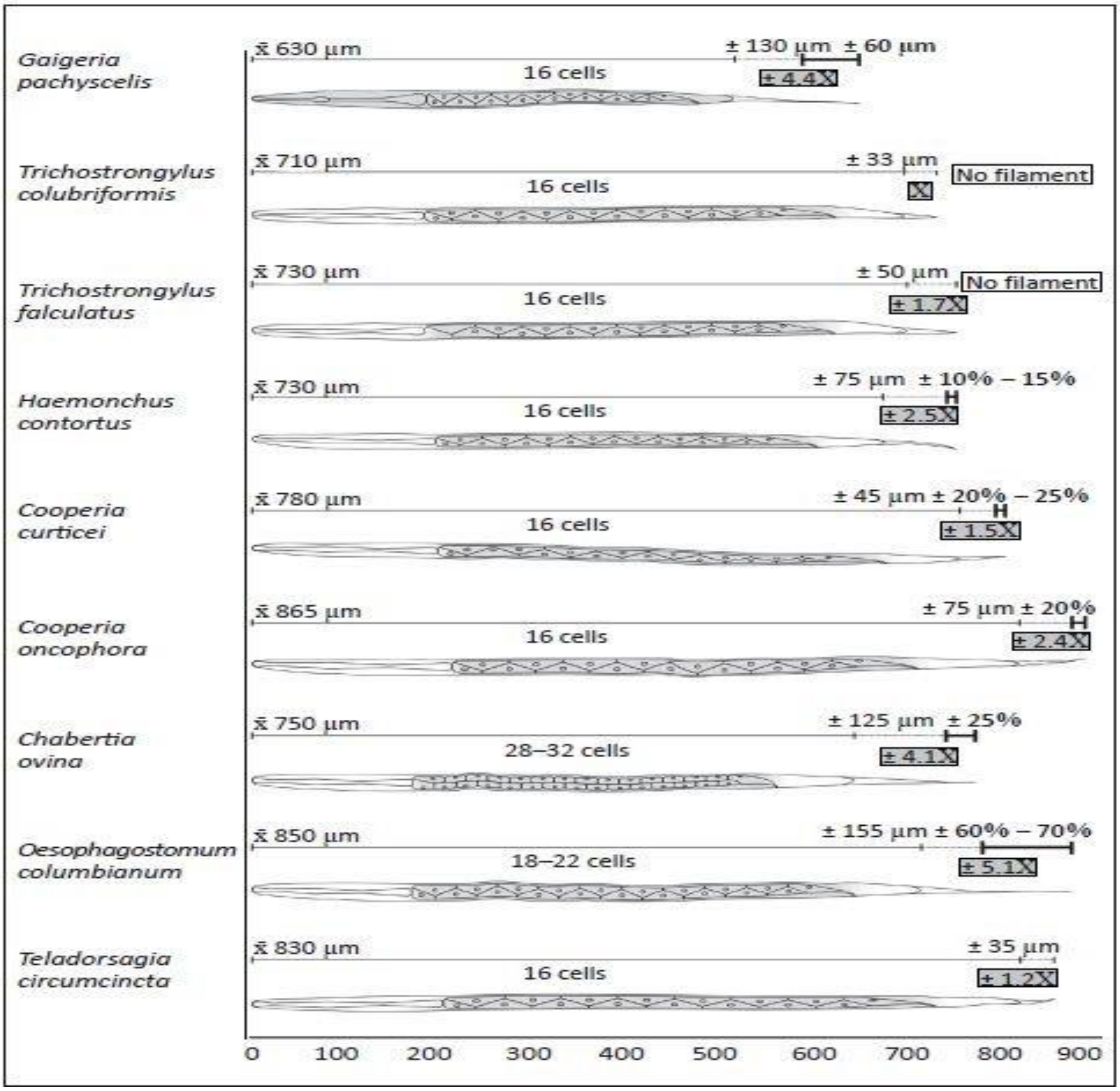

Figure 3.2: Key used to identify the third stage larvae of common nematodes of small ruminants (Adapted from van Wyk and Mayhew 2013)

3.2.3.6 Statistical analysis

Data was analysed using Statistical Analysis System (SAS) and the Pearson’s correlation coefficient (Pearson’s r) was used to measure the linear correlation between between minimum temperature and faecal egg counts, maximum temperature and faecal egg counts, rainfall and faecal egg counts and humidity and faecal egg counts.

3.3 Results

Strongyle-type eggs were the most common and were detected throughout the study period in sheep in Limpopo province, with the highest mean peak egg counts observed in Vhembe district in November 2018 (EPG 1887) and in Waterberg district in November 2017 (EPG 1811), with the highest prevalence of 100%. Figure 3.3 shows that the lowest EPG counts were recorded for Capricorn district (EPG 299) in September 2017 even though prevalence was 82% and conveniently no rainfall was recorded for that particular month.

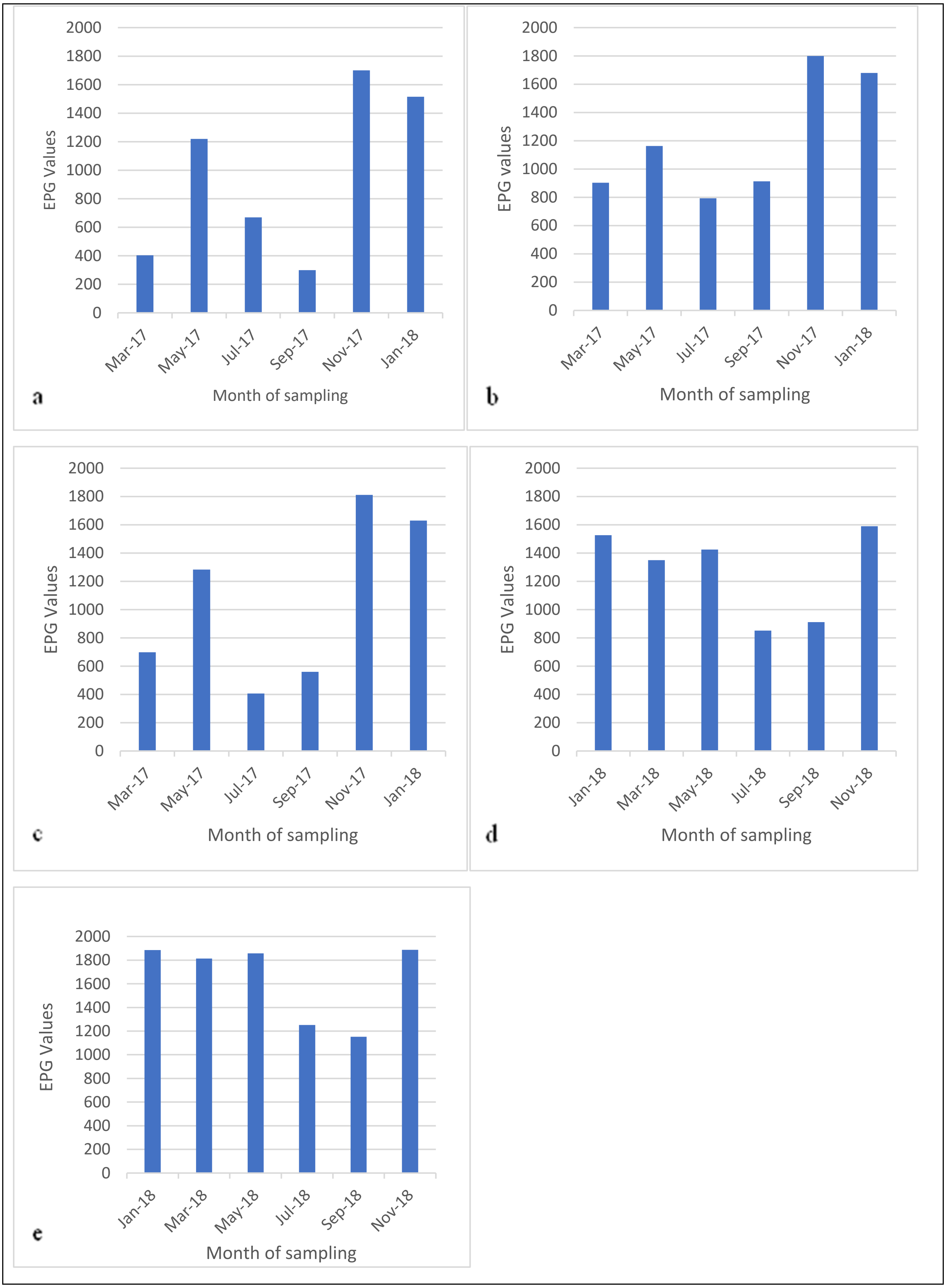
a
EPG Values
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Figure 3.3: Mean egg counts (Egg Per Gram) of gastrointestinal nematodes of sheep in Limpopo (a) Capricorn district, (b) Sekhukhune district, (c), Waterberg district, (d) Mopani district and (d) Vhembe district

The observed decrease in EPG's during the cold dry season did not effectuate statistical difference ($p > 0.05$) in all districts except for Mopani and Vhembe (Table 3.1). During the hot wet season, the sheep examined in Vhembe district revealed FAMACHA© mean score of 4 indicating anaemia. The sheep in all the other districts revealed a mean score of 3 indicating mild anaemia during the hot wet season. On the other hand, the sheep examined in all the districts during the cold dry season were non-anaemic as indicated by the FAMACHA© score of 1 for sheep in the Waterberg and a score of 2 for the animals in all the other districts. Higher EPG's and FAMACHA© scores were observed during the hot wet season and the opposite was seen during the cold dry season indicating that worm burden was responsible for anaemia in sheep (Table 3.1).

Table 3.1: Mean maximum temperature, rainfall, FEC and FAMACHA© scores during hot wet and cold dry seasons

District	Maximum temp		Rainfall (mm)		FEC		FAMACHA© Score	
	Hot wet	Cold dry	Hot wet	Cold dry	Hot wet	Cold dry	Hot wet	Cold dry
Capricorn	26.3^{a}	24.4^{a}	35.8^{a}	0.7^{a}	1210^{a}	485^{a}	3^{a}	2^{a}
Sekhukhune	27.9^{a}	30.6^{a}	36.4^{a}	8.3^{a}	1386^{a}	853^{a}	3^{a}	2^{a}
Waterberg	30.0^{a}	27.5^{a}	44.4^{a}	0.7^{a}	1356^{a}	483^{a}	3^{a}	1^{a}
Mopani	28.3^{a}	26.8^{a}	31.3^{a}	6.6^{a}	1473^{a}	882^{b}	3^{a}	2^{b}
Vhembe	28.0^{a}	26.0^{a}	29.3^{a}	3.3^{b}	1861^{a}	1202^{b}	4^{a}	2^{b}

Means with the same letter within a row ($^{a-b}$) are not significantly different ($p = 0.05$). FEC= Faecal egg counts

High EPG' s counts correlated with the high rainfall experienced during November month in all the districts but particularly high in Waterberg and Capricorn districts with 68.4 mm and 65.2 mm respectively (Figure 3.4). Results also revealed a high nematode prevalence (88 – 99%) in all districts with mean faecal egg counts ranging between 1210 – 1861 eggs per gram (EPG) during the hot wet season. During the cold dry season, prevalence decreased to 75 – 83% with mean egg counts ranging between 453 – 1202 EPG. However, the difference in both nematode prevalence and mean egg counts was not significant ($p > 0.05$) between the hot wet and cold dry seasons.

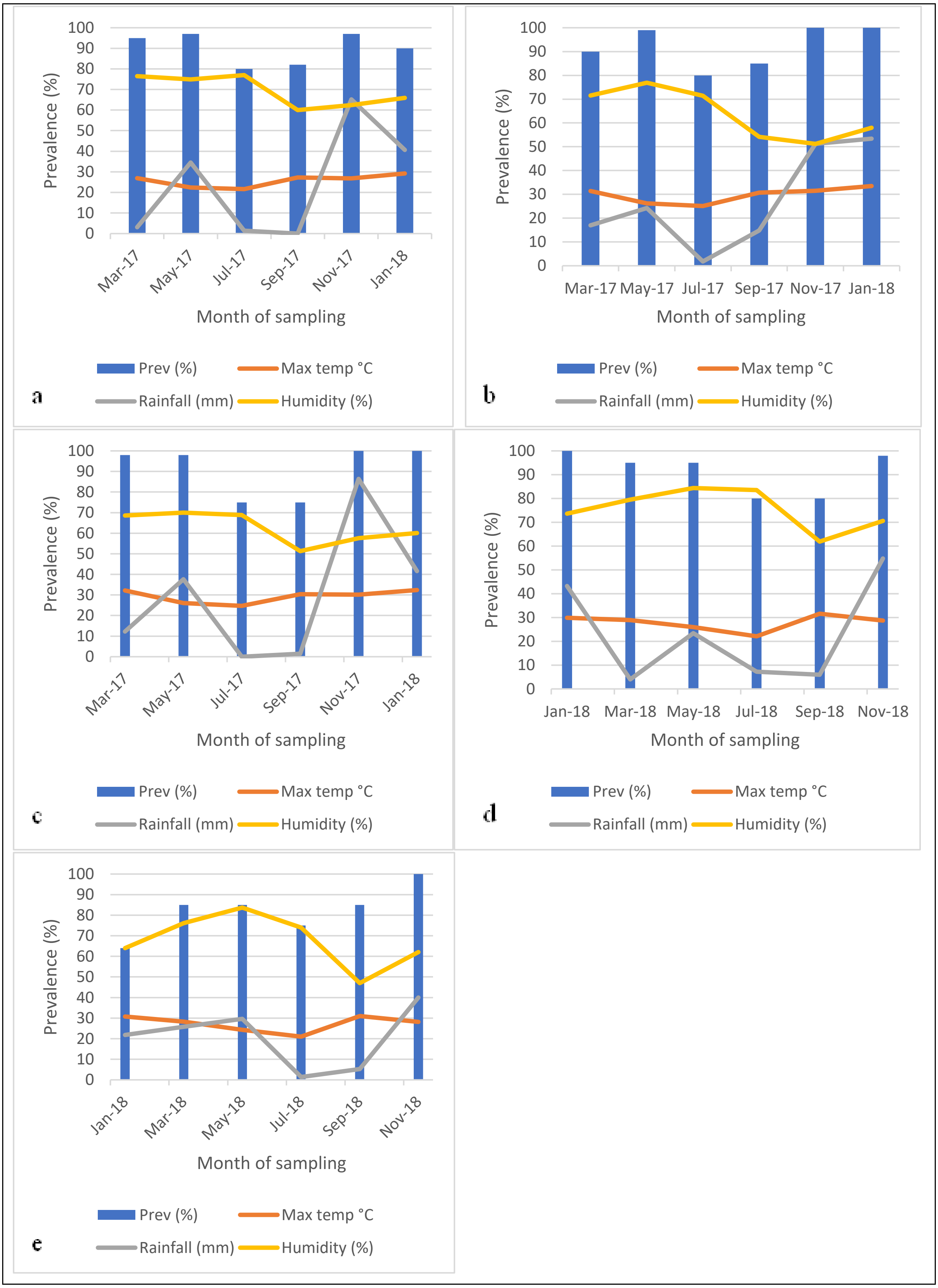

Figure 3.4: Seasonal prevalence of gastrointestinal nematodes of sheep in Limpopo (a) Capricorn district, (b) Sekhukhune district, (c), Waterberg district, (d) Mopani district and (d) Vhembe district

Figure 3.5 shows a positive correlation between mean minimum temperature and mean FEC (r=0.482; p=0.007) and between mean rainfall and mean FEC (r=0.755; p= ≤0. 0001) in all the districts of Limpopo Province.

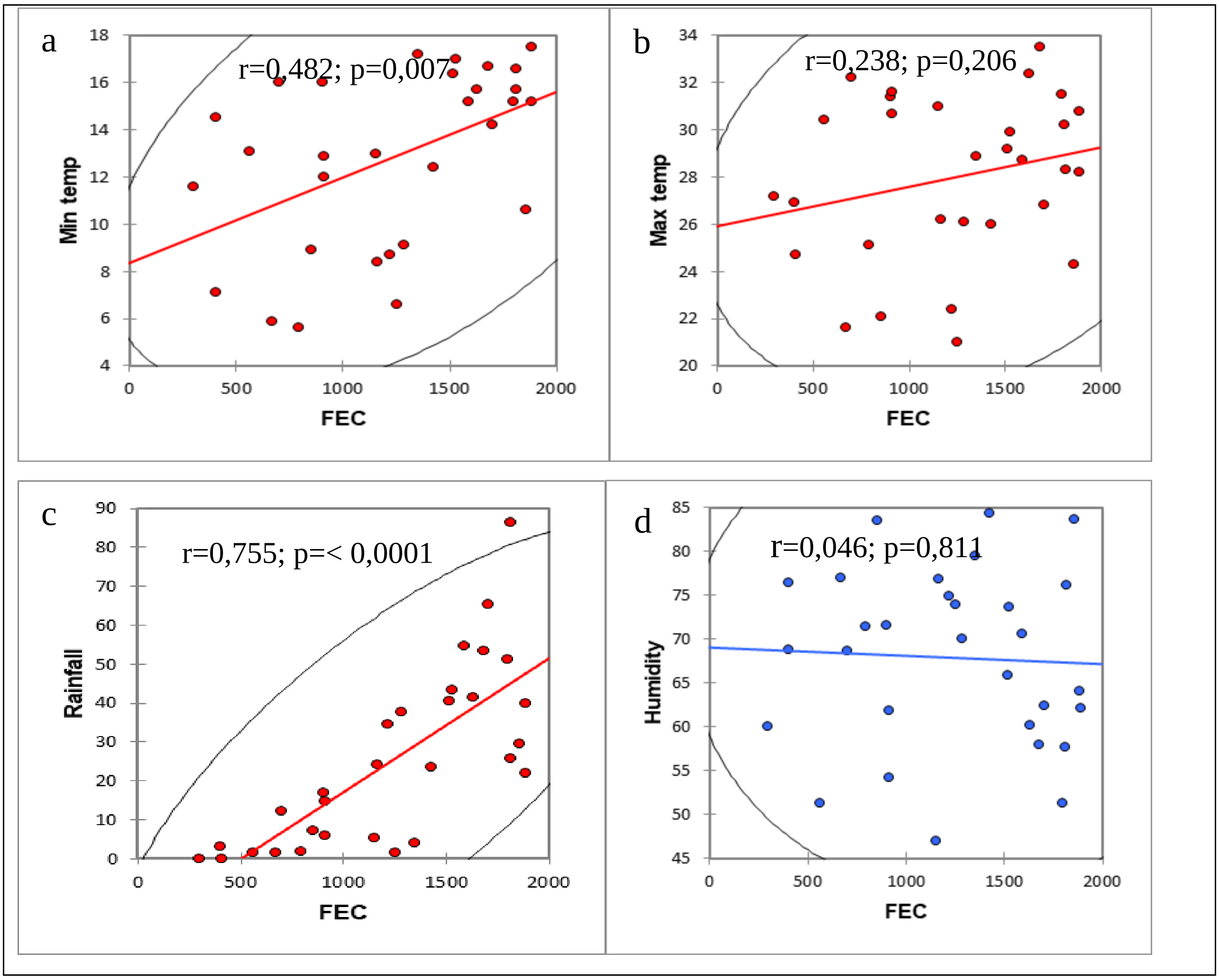

Figure 3.5: Correlation between minimum temperature and faecal egg counts (a), maximum temperature and faecal egg counts (b), rainfall and faecal egg counts (c) and humidity and faecal egg counts (d) in the five districts of Limpopo province

Furthermore, there was also a strong correlation (r = 0.959; p = ≤ 0. 0001) between worm burden and clinical anaemia in sheep (Figure 3.6). However, an overall weak correlation existed between mean humidity and mean FEC (r=0.046; p=0.811) in all the districts.

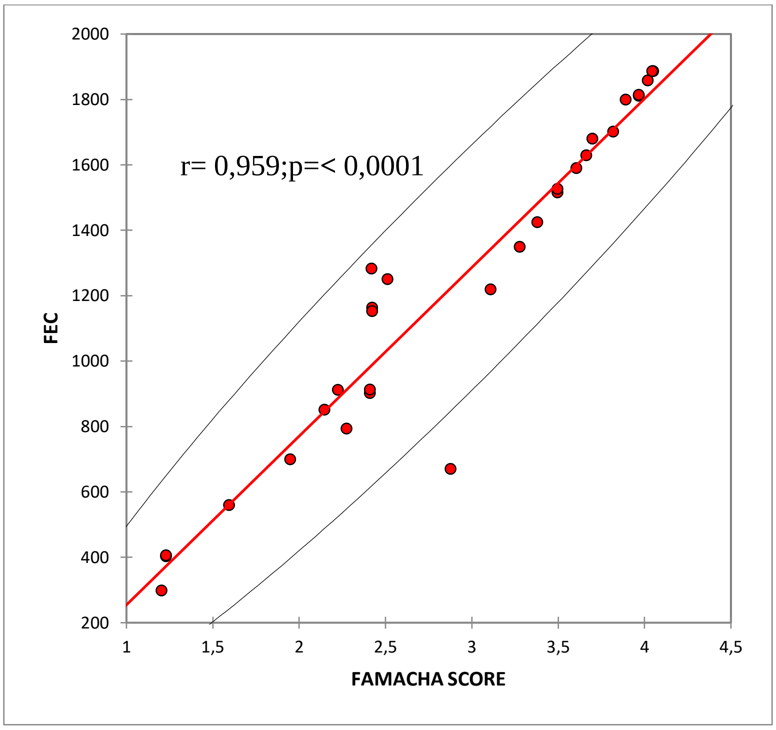

Figure 3.6: Correlation between faecal egg counts (FEC) and FAMACHA© scores of five flocks screened in Limpopo province

Figure 3.7 shows the proportions of GINs genera in Limpopo province at different seasons of the year. Results of the composite faecal cultures revealed 4 species of nematodes in all the five districts of Limpopo province, namely, *Haemonchus contortus*, *Trichostrongylus/Teladorsagia* spp and *Oesophagostomum columbianum. Haemonchus contortus* was the dominant nematode species, representing 70–93% of the total larval recovery throughout the entire study period in all the districts of Limpopo province with the highest percentage of 93% recorded for Mopani in November 2018. This was followed by *Trichostrongylus/Teladorsagia* spp. which represented 5–28% of the total infective larvae harvested from the composite faecal cultures during the study period. *Oesophagostomum columbianum* was detected in small percentages, but never exceeding 5% throughout the study period. Furthermore, the L_3 of *Trichostrongylus* spp. of small ruminants are difficult to differentiate from those of *Teladorsagia* spp. because they are similar in length and can only be differentiated generically by tail morphology after exsheathment and as a result, they were grouped during the count.

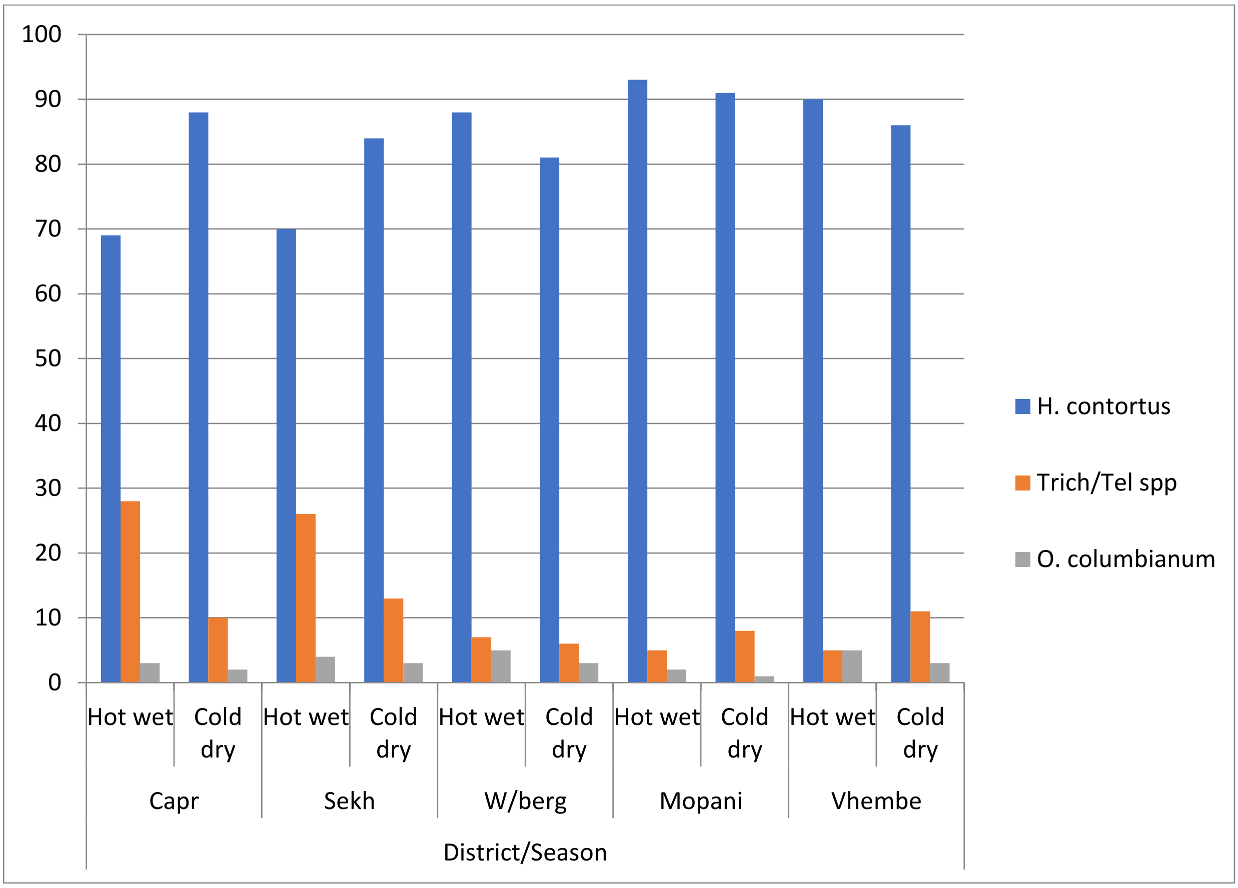

Figure 3.7: Mean percentage of gastrointestinal nematode genera in five districts of Limpopo province during hot and dry season.

3.4 Discussion

High EPG' s counts that correlated with the high rainfall experienced during November month in all the districts followed the well-known pattern influenced primarily by temperature and moisture as they were highest in the hot-wet season and lowest in the cold-dry season. This pattern of infection was also observed in the study conducted by Kumba et al. (2003). The high prevalence of GINs seen in the current study could be due to the availability of suitable climatic conditions such as relatively high temperatures and rainfall that support the prolonged survival and development of larval stages of most nematodes (Rossanigo and Gruner 1995).

The development of GINs larvae in the present study occured in a temperature range of approximately 10–36 °C suggesting that there is a positive correlation between mean minimum temperature and mean FEC and between mean rainfall and mean FEC in all the districts of Limpopo which is in agreement with Getachew et al. (2007). As a matter of fact, the environmental temperatures are always conducive for larval development in most sub-Saharan African countries and South Africa is no exception. (Sissay et al. 2007). Generally, the trend of GINs FECs is associated with the rainfall pattern (Pfukenyi et al. 2007). Results of the present study agree with those of a Zimbabwean study, FECs increase from November and December followed by a decline to low levels during the dry period from May and June (Vassilev 1999; Pfukenyi et al. 2007). In the same breath, the population of infective larvae on pastures increases to a maximum during the hot wet season, which coincides with the peak in FECs (Moyo *et al.* 1997). Furthermore, results of the present study agree with the results of previous studies where there was a positive correlation between EPG level and FAMACHA© scoring in sheep. (Sissay et al. 2007) which could be explained by the dominance of the blood-sucking nematodes such as *Haemonchus* spp. However, the present study was in contrast with the study conducted by Mohammed et al. (2016) that showed no correlation between EPG and FAMACHA© score

Haemonchus contortus were the most dominant nematode followed by *Trichostrongylus/Teladorsagia* spp and *Oesophagostomum columbianum.* These results are concurrent with the results of earlier studies conducted in South Africa on the epidemiology of gastrointestinal nematodes that have also reported *Haemonchus contortus* as the most dominant nematode infecting sheep under commercial farming system (Horak and Louw 1977; Horak 1978) and under small-scale resource-poor farming conditions in South Africa (Tsotetsi and Mbati 2003; Tsotetsi et al. 2013). The observed results, of highest EPGs with the largest percentages of *H. contortus* L_3 in faecal cultures during the rainy seasons, were in accordance with studies in other countries in Africa with distinct rainy and dry seasons (Ghana: Agyei 1991; Zimbabwe: Pandey et al. 1994; Kenya: Nginyi et al. 2001). This can be attributed to the high biotic potential of *H. contortus,* resulting in this parasite rapidly taking up dominance at times when environmental conditions on pasture are ideal for the development and survival of the free-living stages. Furthermore, studies undertaken in other African countries cite *Haemonchus* spp., *Trichostrongylus* spp., *Cooperia* spp. and *Oesophagostomum* spp. as widely encountered strongyle genera of small ruminants (Kanyari et al. 2009). The high biotic potential and shorter generation interval of *Haemonchus contortus* allows for greater contamination of pastures and re-infection of animals (Torres-Acosta et al. 2003) and this probably accounts for its higher prevalence than *Trichostrongylus/Teledorsagia* spp. and *Oesophagostomum columbianum* encountered in this study. In addition to the high biotic potential and shorter generation interval, a mature female of *Haemonchus contortus* can produce 5000–7000 eggs per day while *Trichostrongylus* spp. produce only 100–200 eggs per day; this shows a difference in their reproduction abilities (Getachew et al. 2007). On the other hand, *Oesophagostomum columbianum* has a very long generation interval of 45 days that causes its prevalence to be much lower (Wahab and Adanan 1992). The comprehensive epidemiological knowledge gained through this study about the distribution of GINs in the sheep flocks of resource-poor farmers in Limpopo province. The effect of local climatic factors in Limpopo province on seasonal patterns in L_3 availability is also important for formulation of strategic and appropriate cost-effective deworming programs.

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CHAPTER 4: RISK FACTORS ASSOCIATED WITH OCCURRENCE OF ANTHELMINTIC RESISTANCE IN SHEEP OF RESOURCE POOR FARMERS IN LIMPOPO PROVINCE, SOUTH AFRICA

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Abstract

Anthelmintic treatment is the most common way of controlling nematode infections in ruminants even though several countries have reported anthelmintic resistance (AR), resulting in limitation for sustainable small ruminant production. The aim of the present study was to evaluate the knowledge of resource-poor sheep farmers in Limpopo province of South Africa on the use of anthelmintics. A questionnaire regarding helminthosis control practices was administered to small ruminant farmers in five districts of Limpopo province namely Capricorn, Sekhukhune, Waterberg, Vhembe, and Mopani. A total of 77 resource-poor farmers were interviewed between June and August of 2017 using a structured questionnaire with a combination of qualitative and quantitative open-ended questions. The interviewed farmers were divided into three groups based on their farming experience (< 5; 6–10, and > 10 years of farming experience). Limited farming experience was shown as one of the risks, as farmers that owned sheep for less than 10 years could not identify the symptoms of gastrointestinal parasites infection and did not know how nematodes are transmitted to animals. However, no significant difference ($p < 0.05$) was found to exist between the three groups of farmers in terms of clinical signs identification and correct application of anthelmintics. About 43% of the respondents were unaware of gastrointestinal nematodes (GI) that infect sheep, could not identify the clinical symptoms of gastrointestinal nematodes infection, and only 34% knew how animals become infected. Although 67.5% of farmers mentioned that they never dose their sheep, 32.5% used anthelmintics at varying times of the year. None of the farmers weighed their sheep before dosing them instead visual appraisal of individual weight was the most common means of estimating the anthelmintic dose. The above information is an indication of risks associated with possible occurrence of anthelmintic resistance in the study areas. There is therefore, a need to train resource-poor farmers of small stock on proper application of anthelmintic treatment and to educate them on how to prevent development of AR. Future studies on AR should also be conducted in the province in flocks with high-treatment frequencies to establish the occurrence of AR using both in vivo and in vitro methods. The most common risk factor associated with the occurrence of AR in all the five districts of Limpopo province was found to be the use of anthelmintics without weighing the animals to determine the correct dosage.

Keywords: Gastrointestinal nematodes; Anthelmintic resistance; Visual appraisal; Resource-poor farmers; Limpopo province

4.1 Introduction

Gastrointestinal nematodes of small ruminants are distributed worldwide and are particularly important parasites of ruminants in all regions across the tropical and sub-tropical countries (Martinez-Valladares 2013). These infections cause low productivity due to stunted growth, poor weight gain, feed utilization, feeding and water intake, lower meat, wool and milk production, cost of treatment and mortality in young animals (Mohammed et al. 2016). Anthelmintic treatment is the most common way of controlling nematode infections in ruminants even though several countries have reported anthelmintic resistance (AR), representing a limitation for sustainable small ruminant production (Domke et al. 2012). Antimicrobial resistance also ranks as one of the most important global health concerns of our age and threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi (WHO 2014).

Resistance against drugs belonging to the same anthelmintic drug class is called side-resistance, whereas cross and multidrug-resistance refers to resistance against two or multiple drugs belonging to different anthelmintic drug classes (Torres-Acosta et al. 2012). Development of AR can be limited by ensuring that the nematode parasites are exposed to an effective anthelmintic drug concentration and to consider the timing and frequency of anthelmintic drug treatments so that only a small proportion of the population is exposed to the anthelmintic (Sargison 2016).

The AR is a problem in countries such as Australia, New Zealand, South Africa, and many Latin American countries (Dolinská et al. 2012; Torres-Acosta et al. 2012). In Europe, anthelmintic resistance has been reported in the Slovak Republic (Čerňanská et al. 2006), Spain (Alvarez-Sanchez et al. 2001), Italy and Greece (Geurden et al. 2014), the United Kingdom (Taylor et al. 2009), and the Netherlands (Borgsteede et al. 2007). According to Van Wyk et al. (1997), 99% of South African sheep farms harbour nematode strains resistant to at least one class of anthelmintics and 40% of South African farms were found to house strains of nematodes resistant to three or more classes of anthelmintics. Van Wyk et al. (1999) reported that South Africa was possibly the highest country in the world with AR of *H. contortus* and with emerging strains that could soon be impossible to treat with any of the existing anthelmintics. AR have been reported in both commercial (Van Wyk 1999; Van Wyk 2001) and resource-poor farming (Bakunzi 2003; Tsotetsi et al. 2013) sectors, with the commercial sector being described as the worst in the world (Vatta and Lindberg 2006). Table 4.1 shows a detailed account of AR evaluated by the Faecal Egg Count Reduction Test (FECRT) in small ruminants in South Africa.

Table 4.1: Anthelmintic resistance evaluated by the Faecal Egg Count Reduction Test (FECRT) in small ruminants in South Africa

Area resistance reported	**Drug found to have developed resistance**	**Host animal**	**Source**
Limpopo province, Tompi Seleka	Benzimidazole and Rafoxanide	Sheep	Van Wyk et al. (1999)
Northwest Province, Mafikeng	Rafoxanide, Levamisole and Fenbendazole	Goats	Bakunzi (2003)
Mpumalanga, Emerlo	Benzimidazole, Levamisole, Ivermectin and Rafoxanide	Sheep	Van Wyk et al. (1999)
Kwazulu Natal	Benzimidazole, Levamisole, Ivermectin and Rafoxanide	Sheep	Van Wyk et al. (1999)
Gauteng Province, Hammanskraal	Albendazole, Levamisole and Ivermectin	Goats	Tsotetsi et al. (2013)
	Levamisole and Ivermectin	Sheep	Tsotetsi et al. (2013)
Gauteng province, Nigel	Albendazole, Levamisole and Ivermectin	Goats	Tsotetsi et al. (2013)
	Albendazole and Ivermectin	Sheep	Tsotetsi et al. (2013)

The most commonly used anthelmintic classes are macrocyclic lactones, benzimidazoles and imidazothiazoles (Cezar et al. 2010). Many reports of AR are cases of benzimidazoles or levamisole, but the number of cases of resistance to the macrocyclic lactone, namely, ivermectin is increasing (Papadopoulos 2008). Reports of resistance to doramectin and moxidectin are less common (Papadopoulos et al. 2012).

The risk of under dosing and a continued use of one class of anthelmintics, irrespective of efficacy status are frequently encountered factors enhancing development of anthelmintic resistances (Čerñanská et al. 2008; Aga et al. 2013). The aim of the present study was to evaluate the resource poor farmer's knowledge on anthelmintic use in Limpopo province, South Africa.

4.2 Objective of the study

To evaluate the resource poor farmer's knowledge on anthelmintic use using questionnaire survey.

4.3 Materials and methods

4.3.1 Study area description

This study focused on Limpopo Province located in the north of South Africa (Figure 3.1). It is one of the developing provinces in South Africa and is particularly vulnerable to climate change impacts, due to its exposure to extreme weather events (Cook et al. 2004). The province has three distinct climatic regions: The Lowveld region which is characterized by a semi-arid climate(s), the Middle- and Highveld that is considered semi-arid, and Escarpment that experiences sub-humid climate (Limpopo Department of Agriculture 2008).

The province experiences long sunny days and dry weather conditions on most days. During the summer months, which extends from November to January, warm days are often interrupted by a short-lived thunderstorm (Limpopo Department of Agriculture, 2008). It can get very hot in October to March, with average temperatures rising to 27ºC in summer and 20ºC in winter. The bulk of the precipitation occurs in summer, and annual rainfall totals range from about 400 - 600 mm over most of the province (Anon 2007). Limpopo is a province having a high number of rural dwellers dependent on natural resources and farming is of considerable importance (Thomas et al. 2005). In the study areas where the questionnaire was distributed, the flocks were constituted mainly of Pedi sheep and cross breeds. The animals were kraaled at night and released to the communal grazing areas in the morning with no supplementary feeding provided.

4.3.2 Questionnaire survey

A questionnaire (Appendix 1) regarding helminthosis control practices was administered to small ruminant farmers in five districts of Limpopo province, namely Capricorn, Sekhukhune, Waterberg, Vhembe and Mopani. A total of 77 resource-poor farmers (farmers who have a history of dependency on state-provided services such as dipping) were interviewed between June and August of 2017 using a structured questionnaire with a combination of qualitative and quantitative, open-ended questions. Participant farmers were derived from the extension officers and animal health technician's data base in Limpopo province and they owned 40 - 100 sheep.

The questionnaire was composed of two sections, whereby the first section was related to main characteristics of the farm management such as flock size, breed, grazing conditions and housing time. The second section was dedicated to helminth parasite control practices. Questions documented information on mode of infection, clinical signs linked to parasitism, time and reason for anthelmintic treatment, anthelmintic products used, dose determination and mode of application. The farmers in Capricorn, Sekhukhune and Vhembe districts converged at the dipping tanks with the exception of Waterberg and Mopani districts where farmers were visited in their respective homes. All the farmers were interviewed individually.

4.3.3 Statistical analysis

Data collected was manually coded and analysed using descriptive statistics and frequencies. Microsoft® Excel 2016 and SAS Statistics (Version 9.4) statistical package were also used to analyse the data. Chi-square analysis was used to compare the proportions of yes to no in response to different variables.

4.4 Results

All 77 questionnaires were returned, and demographics revealed that 88% of the respondents were older people aged over 40 years, of which 50 (73.5%) were males and 18 (26.4%) were females. The results presented in Table 4.2 show a numerical difference with no significant difference ($p > 0.05$) between the male and the female farmers in terms of knowledge on the risk factors that are associated with the development of anthelmintic resistance.

Table 4.2: The percentages of yes between male and female on questions relating to risk factors associated with development of anthelmintic resistance

Risk factors	**Female**	**Male**	**Chi square value**	**Pr > Chi square**
Knowledge of symptoms of infection	15.91	84.09	2.27	*0.13
Knowledge of mode of infection	15.38	84.62	1.02	*0.31
Usage of anthelmintics	15.63	84.37	1.32	*0.24
Knowledge of dosage calculation	17.39	82.61	0.41	*0.51

*Pr > Chi square = Probability at which the proportions of yes are different

The highest number of female farmers was found in Sekhukhune district (38.8%) and the lowest was in Waterberg and Capricorn districts with 2 (11.1%) female farmers each. There was no significant difference (p > 0.05) between the farmers falling in different age group categories (21 - 30; 31 - 40) in respect of their knowledge of symptoms of infection, knowledge of mode of infection, usage of anthelmintics and knowledge of dosage calculation.

The results indicated that visual appraisal of individual weight was the most common means (100%) of estimating the anthelmintic dose used in sheep. Table 4.3 shows that only a small percentage (4.35%) of farmers from Vhembe district stated that they know how to calculate anthelmintic dose as compared to the farmers in Capricorn, Mopani, Sekhukhune and Waterberg districts at 30.43, 34.78, 13.04 and 17.39 respectively.

Table 4.3: The percentages of yes from farmers in the five districts of Limpopo province on questions relating to risk factors associated with development of anthelmintic resistance

Risk factors	Capr	Mop	Sekh	Vhe	W/berg	Chi square value	Pr > Chi square
Knowledge of symptoms of infection	27.27	25.00	29.55	4.55	13.64	24.83	*<.0001
Knowledge of mode of infection	43.31	19.23	19.23	7.69	11.54	16.10	*0.0029
Usage of anthelmintics	25.00	31.25	18.75	12.50	12.50	13.83	*0.0079
Knowledge of dosage calculation	30.43	34.78	13.04	4.35	17.39	17.51	*0.0015

Capr = Capricorn; Mop = Mopani; Sekhu = Sekhukhune; Vhe = Vhembe; W/berg = Waterberg. *Pr > Chi square = Probability at which the proportions of yes are different

These findings correspond with a similarly small percentage (4.55%) of farmers in Vhembe with some knowledge of symptoms of internal parasites infection and the probability at which the proportions of yes are different was highly significant ($p < 0.001$).

The results of the present study revealed that 43% of the farmers were not aware of gastrointestinal nematode (GI) that infect sheep and again 66% did not know how animals become infected. The results in Table 4.4 reveals that the farmers that owned sheep for more than 10 years were better informed than their inexperienced counterparts in terms of identifying symptoms of internal parasites infection and knowledge of infection mode. However, no significant difference ($p > 0.05$) was found to exist between the three groups of farmers regarding the usage and correct application of anthelmintics.

Table 4.4: The percentages of yes between experienced and inexperienced farmers in Limpopo province on questions relating to risk factors associated with development of anthelmintic resistance

Risk factors	Less than 5 yrs.	6 - 10 yrs.	More than 10 yrs.	Chi square value	Pr > Chi square
Knowledge of symptoms of infection	15.91	20.45	63.64	5.21	*0.07
Knowledge of mode of infection	7.69	23.08	69.23	6.55	*0.03
Usage of anthelmintics	18.75	18.75	62.50	1.26	*0.53
Knowledge of dosage calculation	21.74	17.39	60.87	0.27	*0.87

*Pr > Chi square = Probability at which the proportions of yes are different

The farmers with a lower level of education had a better awareness of three out of the four risk factors that are associated with the development of anthelmintic resistance than those with matric qualification (Table 4.5).

Table 4.5: The percentages of yes between farmers of different education levels in Limpopo province on questions relating to risk factors associated with development of anthelmintic resistance

Risk factors	Below matric	Post matric	Chi square value	Pr > Chi square
Knowledge of symptoms of infection	63.64	36.36	3.05	*0.08
Knowledge of mode of infection	57.69	42.31	3.62	*0.05
Usage of anthelmintics	53.13	46.87	8.98	*0.002
Knowledge of dosage calculation	52.17	47.83	5.95	*0.01

*Pr > Chi square = Probability at which the proportions of yes are different

About 67.5% of resource poor sheep farmers in Limpopo province never use anthelmintics although 32.4% of them had deworming schedule for their sheep. However, the Chi square analysis of the five districts of Limpopo province yielded a significant statistical difference ($p < 0.05$) for farmers' usage of anthelmintics and knowledge of dosage calculation (Table 4.5).

Results of the present study also revealed that 43% of the respondents cannot identify the clinical symptoms of gastrointestinal nematodes infection. However, loss of body condition and rough coat were mentioned 11 times (31.4% frequency) and 10 times (28.5% frequency) respectively as clinical signs of gastrointestinal infection (Table 4.6).

Table 4.6: Knowledge on clinical manifestation/signs of gastrointestinal infection

Clinical symptoms	**Frequency**	**Frequency %**
Nasal discharge	6	17.1
Rough coat	10	28.5
Loss of body condition	11	31.4
Worms of in the faeces	3	8.5
Diarrhea	3	8.5
Bottle jaw	2	5.7

Forty-eight percent of the respondents know the infection occurrence months, with 65% of them mentioning summer months when it is hot and wet, 19% citing winter and small percentages (8%, 5% and 3%) saying infections occur during spring, all year round and at the beginning of both winter and summer respectively (Figure 4.1).

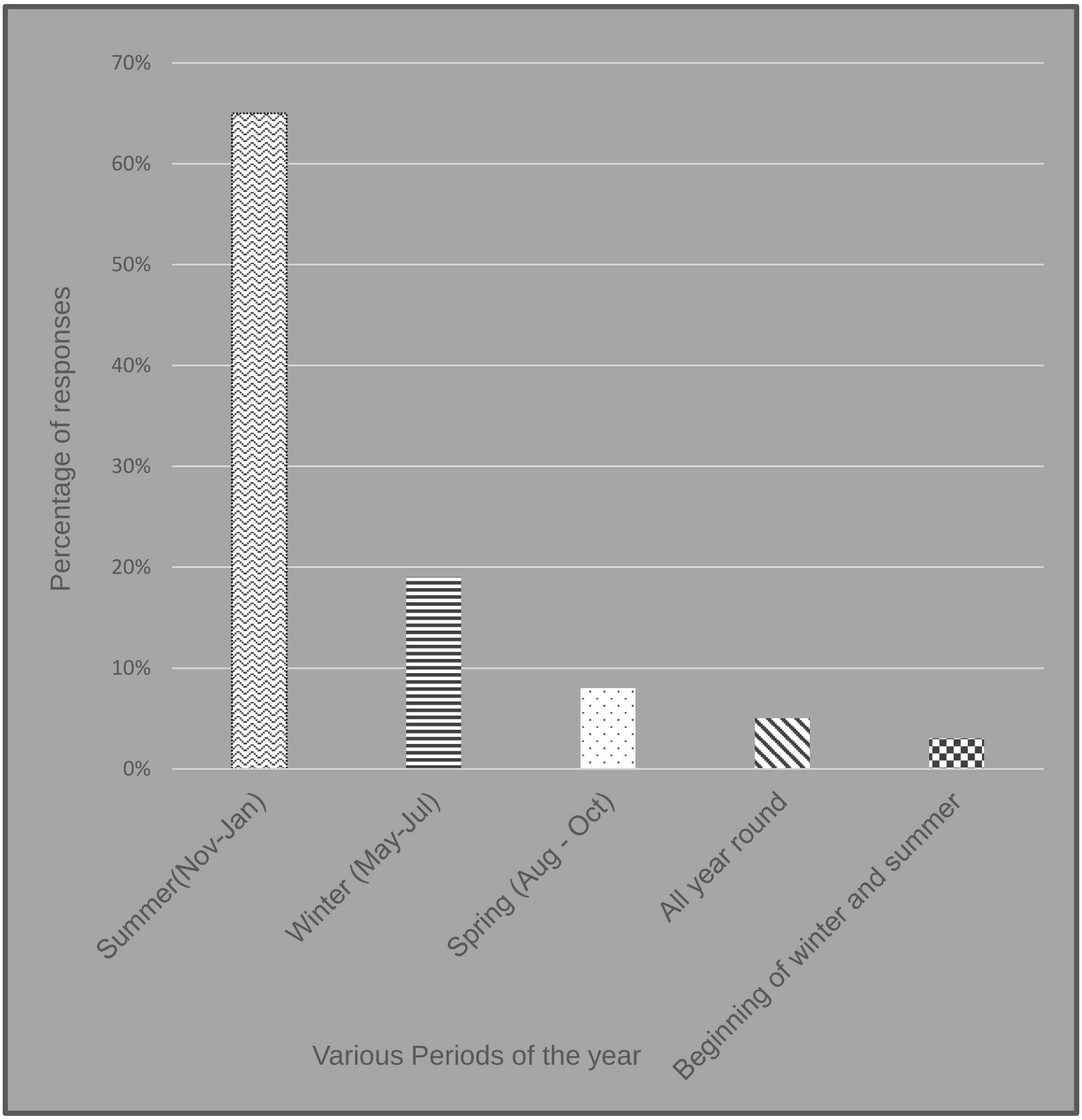

Figure 4.1: Periods of helminth infections according to resource-poor farmers' knowledge in Limpopo province

Table 4.7 shows that 67.5% of the farmers never use commercial anthelmintics whilst 32.5% apply anthelmintics at varying intervals during the year.

Table 4.7: Helminths control practices of resource poor farmers in Limpopo province

Helminth control factor	Frequency	Frequency %
Anthelmintic class		
Benzimidiazole	26	70.3
Macrolytic lactones	9	24.3
Levamisole	1	2.7
Praziquantel + Levamisole + Oxfendazole + Abemectin	1	2.7
Treatment frequency		
Once per year	3	3.9
Twice per year	9	11.7
Three times in a year	13	16.8
Never	52	67.5
Frequency of active AI change (Months)		
3 months	10	66.67
4 months	2	13.33
6 months	3	20.00

Where AI = Active ingredient

Benzimidazoles were the most common anthelmintic class used in sheep. Macrocyclic lactones were mentioned 9 times (24.3% frequency) as the preferred drug for the control of gastrointestinal nematodes in sheep with levamisole and praziquantel levamisole combination mentioned once for each (Table 4.7).

4.5 Discussion

Research, particularly in the UK, Australia, South Africa and New Zealand, has resulted in a growing awareness of the problems of anthelmintic resistance and recognition of potential resistance-delaying strategies, which can be used on farms (Van Wyk 2001). The main factors for selection for anthelmintic resistance are, a high treatment frequency (Vijayasarathi et al. 2016), under-dosing and the use of the same anthelmintic class over several years (Sargison et al. 2007; Sangster et al. 2018). These factors, alone or in combination, together with certain types of farm management can accelerate the development of AR (Jackson 2009). Anthelmintic drugs currently used in SA include benzimidazole (Valbazen®, Zoetis, United States), macrocyclic lactones (Ivomec®, Merial, United States), levamisole (Tramisol Ultra®, Coopers and Intervet,) Praziquantel + Levamisole + Oxfendazole + Abemectin combination (Triple A plus, Virbac, New Zealand). Generally, the misuse of drugs has been observed even outside the scope of treatment of gastrointestinal nematodes. Dagnachew et al. (2017) reported that trypanocidal treatment frequencies are higher and injections were mainly given by farmers' untrained personnel.

None of the resource-poor farmers in the present study weighed their sheep before dosing but relied solely on the visual appraisal of an animal to determine its weight and weight estimation may be a cause of under-dosing in Limpopo province. Underestimation of real weight has potential to lead to under-dosing, which can contribute to the development of AR (Chartier et al. 2001; Kupčinskas et al. 2016, Sangster et al. 2018). In South Africa, Bakunzi (2003) also reported under-dosing as one risk factor for AR development. The results of that study attributed AR observed in goats to under-dosing caused by visual appraisal of an animal to estimate its weight as opposed to the actual weighing before dosing to determine the correct anthelmintic dosage.

It is not only the incorrect estimation of animal live-weight that causes under-dosing but also incorrect calibration of drench guns. In consideration of ensuring a correct dose, one has to estimate the weight as accurately as possible, preferably by an individual weighing of each animal (Wolstenholme et al. 2004; McMahon et al. 2017). Results of the current study indicated that only 32.4% of resource poor farmers had deworming schedule for their animals. Although frequency of treatments with anthelmintics varied among farmers, 16.8% of them treated their animals three times a year and these results are in agreement with the result in an Ethiopian survey that showed only 17.78% of the farmers had deworming schedule and 68.33% of the respondents did not deworm their animals (Belina et al. 2017).

Most of the farmers (67.5%) in the current study never treat their animals with anthelmintics. However, there is still a percentage of farmers (32.4%) made up of those farmers who treat their animals once a year (3.9%), twice a year (11.7%) and three times a year (16.8%) with anthelmintics. Those farmers who treat their flocks two or more times per year, do so without coprological checking for the necessity of treatment or its efficacy afterwards and that puts the flocks at risk of anthelmintic resistance.

In another South African study conducted by Tsotetsi et al. (2013), in Gauteng province, the results showed that 88% of the farmers were aware of veterinary helminthosis which is much higher as compared to the 57% of farmers in Limpopo province. On the contrary, a study conducted by Belina et al. (2017) in Ethiopia revealed that 83.61% of the respondents had poor to no information on anthelmintic drugs utilization practices and 78.89% of interviewed respondents did not know the issue of anthelmintics resistance and its future impact.

The majority of resource poor sheep farmers in Limpopo province still had poor understanding on GI nematode infection which is in line with the results of parasite control practices on gastrointestinal nematode in two separate Ethiopian studies (Aga et al. 2013). Contrary to their lack of understanding on gastrointestinal nematode infection, the farmers had knowledge of clinical symptoms and loss of body condition was cited more than any other symptom which was consistent with the results of an earlier study that reported that GI nematode infections was higher in animals with poor body condition (Mohammed et al. 2015). This could be explained by the fact that loss of body condition could be due to factors such as parasitic infections which lead to lower immunological response against infective stage of the parasites (Mohammed et al. 2015). Likewise, in is important to note that as a ruminant, sheep have evolved to utilize relatively poor-quality feed and are often farmed in marginal environments, subject to seasonal variation and/or farming systems with little-to-no supplementation and under these environments and farming systems, sheep can be subjected to both short and long periods of under nutrition which can negatively impact performance (Ferguson et al. 2017).

The results of the present study also showed that a 67.5% of sheep farmers in Limpopo province never use anthelmintics which in itself pre-determines lower risk of AR in

Limpopo Province South Africa. That is a notion supported by earlier workers that higher treatment frequencies increase the selective advantage for resistant parasites, allowing for an increase in the proportion of resistant parasites over time (Sargison 2011) but contrary to the results of the study by Van Wyk et al. (1999) in Limpopo province that revealed AR was still a problem even in sheep flocks where anthelmintic treatment was less intensive. Although a high risk of resistance is eliminated by the large number of farmers that never use anthelmintics in their flocks, some degree of risk may still exist on some farms that use commercial anthelmintics because the survey also revealed that knowledge of AR, the prevalence of gastrointestinal nematodes and proper usage of anthelmintics is lacking. Similar to the findings of studies conducted in the southern part of Ethiopia and Cuba, the present study established that benzimidazoles group of anthelmintics especially albendazole was the most commonly used by resource poor farmers in Limpopo province to deworm their sheep (Kumsa et al. 2010). On the other hand, levamisole was found to be used very rarely in South Africa, as in many European countries (Čerñanská et al. 2008).

Results of the current study are ironic since literature suggests that South Africa has a large proportion of extension officers with an average of 1:487 extension officer to farmer ratio, probably one of the most favourable ratios in Africa. These figures compare favourably with those of developed countries and theoretically, in South Africa, most farmers would be assumed to have contact with extension officers (Akpalu 2013).

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CHAPTER 5: ANTHELMINTIC RESISTANCE IN GASTROINTESTINAL NEMATODES OF SHEEP IN LIMPOPO PROVINCE, SOUTH AFRICA

Abstract

The aim of this study was to confirm occurrence of anthelmintic resistance (AR) in nematodes of sheep in five districts (Capricorn, Mopani, Sekhukhune, Vhembe, Waterberg) of Limpopo Province using *in vivo* Faecal Egg Count Reduction Test (FECRT) and *in vitro* Egg Hatch Test (EHA) and Micro Agar Larval Development Test (MALDT). Forty sheep from flocks with high treatment frequencies on each of the five districts were divided into three treated groups and one untreated control group. Animals were treated with ivermectin (Ivomec®) at 0.2 mg/kg bw, levamisole (Tramisol Ultra®) at l5 mg/kg bw and albendazole (Valbazen®) at 7.5 mg/kg bw. EHA was used to determine AR against thiabendazole (TBZ) and MALDT was used for both TBZ and levamisole (LEV). FECRT detected AR against all the anthelmintic classes in all the 5 districts of Limpopo province except for Sekhukhune where AR was suspected against LEV and ML. Furthermore, AR was also suspected against ML in Capricorn district. EHA results showed AR against TBZ in all districts whilst the MALDT showed no AR against LEV in all the five districts, but detected AR against TBZ in Sekhukhune, Capricorn and Waterberg districts. There was no correlation between FECRT and MALDT in detecting LEV resistance in all the districts. A strong correlation existed between FECRT and EHA as both tests confirmed the occurrence of AR in all the districts except for LEV in Sekhukhune. *Haemonchus* contortus was the most dominating resistant nematode genera identified.

Keywords: Anthelmintic resistance; FECRT; EHA; MALDT; Limpopo province; South Africa

5.1 Introduction

Infections caused by gastro-intestinal nematodes (GINs) in the extensive system of livestock can cause serious economic setbacks (Demeler et al. 2009). This fact can be correctly linked to impaired animal health due to the presence of GINs, resulting in decreased production of meat and milk. Parasitological surveys carried out in other countries such as, Ethiopia and Kenya reported that helminthoses and haemonchosis were the leading causes of mortality among ruminants and small stock in particular (Kagira and Kanyari 2001; Sissay et al. 2007; Kanyari et al. 2009). To control these infections, anthelmintic drugs have been used for almost 4 decades (Geurden et al. 2015).

Conventional methods which include the use of anthelmintics such as benzimidazoles, macrocyclic lactones, and nicotinic agonists have been used to treat GINs but decades of overuse and misuse of these drugs has led to the development of anthelmintic resistance (AR), which has escalated to become a global problem (Kaplan and Vidyashankar 2012).

Anthelmintic resistance has been reported for all anthelmintic classes currently available (Table 5.1), namely benzimidazoles (e.g. flubendazole, albendazole, and fenbendazole), imidazothiazoles (e.g. levamisole), and macrocyclic lactones (e.g. ivermectin) (Kaminsky et al. 2008; Leathwick 2012). In South Africa, AR has been reported in sheep (Van Wyk et al. 1999; Tsotetsi et al. 2013) and goats (Vatta et al. 2001; Bakunzi 2003; Tsotetsi et al. 2013) in both the commercial and resource-poor farming systems. The overall prevalence of AR in South Africa and elsewhere in Africa has, however, not been extensively investigated. The aim of this study was to detect AR in gastrointestinal nematodes of sheep in selected flocks in Limpopo Province using both *in vivo* (Faecal Egg Count Reduction Test [FECRT]) and *in vitro* methods including Egg Hatch Assay (EHA) and Micro Agar Larval Development Test (MALDT).

Table 5.1: Cases of anthelmintic resistance reported in sheep in South Africa and elsewhere

Country	**Anthelmintic (Class)**	**Nematode genera**	**Reference**
South Africa	Levamisole, Morantel	*Trich/Tel spp.*	Van Wyk et al. (1990)
South Africa	Benzimidazole, Fenbendazole, Rafoxinide, Levamisole (BZ, SCL, IMID)	*Haemonchus spp.*	Van Wyk et al. (1999)
South Africa	Albendazole, Closantel, Ivermectin, Levamisole (BZ, SCL, AVM, IMID)	*Haemonchus spp, Trich/Tel spp. Oesophagostomum spp.*	Bakunzi et al. (2013), Tsotetsi et al. (2013)
Zimbabwe	Fenbendazole, Albendazole, Oxfendazole, Levamisole (BZ, IMID)	*Haemonchus spp, Cooperia spp.*	Mukaratiwa et al. (1997)
Zimbabwe	Fenbendazole, Levamisole, Rafoxanide (BZ, IMID, SCL)	*Haemonchus spp.*	Boersema and Pandey (1997)
Zambia	Ivermectin, Albendazole (AVM, BZ)	*Haemonchus spp.*	Gabriel et al. (2001)

Kenya	Ivermectin, Fenbendazole (AVM, BZ)	*Haemonchus spp, Trich/Tel spp. Oesophagostomum spp.*	Mwamachi et al. (1995)
Germany	Levamisole, Ivermectin (IMID, AVM)	*Trich/Tel spp.*	Voigt et al. (2012)
Norway	Albendazole (BZ)	*Trich/Tel spp.*	Domke et al. (2012)
Northern Ireland	Benzimidazole, Moxidectin, Avermectin, Levamisole (BZ, MLB, AVM, IMID)	*Trich//Tel spp, Cooperia spp.*	McMahon et al. (2013)
Switzerland	Avermectin (AVM)	*Haemonchus spp, Trich/Tel spp.*	Artho et al. (2007)
Brazil	Ivermectin (AVM)	*Haemonchus spp.*	Fortes et al. (2013)
India	Fenbendazole, Benzimidazole, Thiabendazole, Tetramisole (BZ, IMID)	*Haemonchus spp, Trich/Tel spp.*	Rialch et al. (2013), Swarnkar and Singh (2011)

Benzimidazoles – BZ; Macrocyclic lactones – ML (Avermectines – AVM or Milbemycin – MLB); Nicotinic agonists (Imidazothiazoles – IMID or Tetrahydropyrimidines – TETR); Aminoacetonitriles derivatives – AAD; Salicylanilides – SCL; *Tel = Teladorsagia; Trich = Trichostrongylus*.

5.2 Objective of the study

To detect anthelmintic resistance of three most widely used drugs against gastrointestinal nematodes of sheep using faecal egg reduction tests and in vitro methods.

5.3 Materials and Methods

5.3.1 Selection of farm animals and sampling

The study was conducted on selected small-scale farms located in five districts of Limpopo Province: Capricorn, Sekhukhune, Waterberg, Mopani and Vhembe (Figure 5.1) of which the participant farmers were derived from the extension officers and animal health technicians’ data base.

5.3.2 Ethics clearance

The study was approved by the scientific committee of Integrated Pest Management, North-West University, with reference no: NWU-01252-19-A9 and research approval in terms of section 20 of the Animal Diseases Act (35 of 1984) from the Department of Agriculture, Forestry and Fisheries (DAFF) was granted. Permission to collect study samples was granted by participating resource-poor farmers.

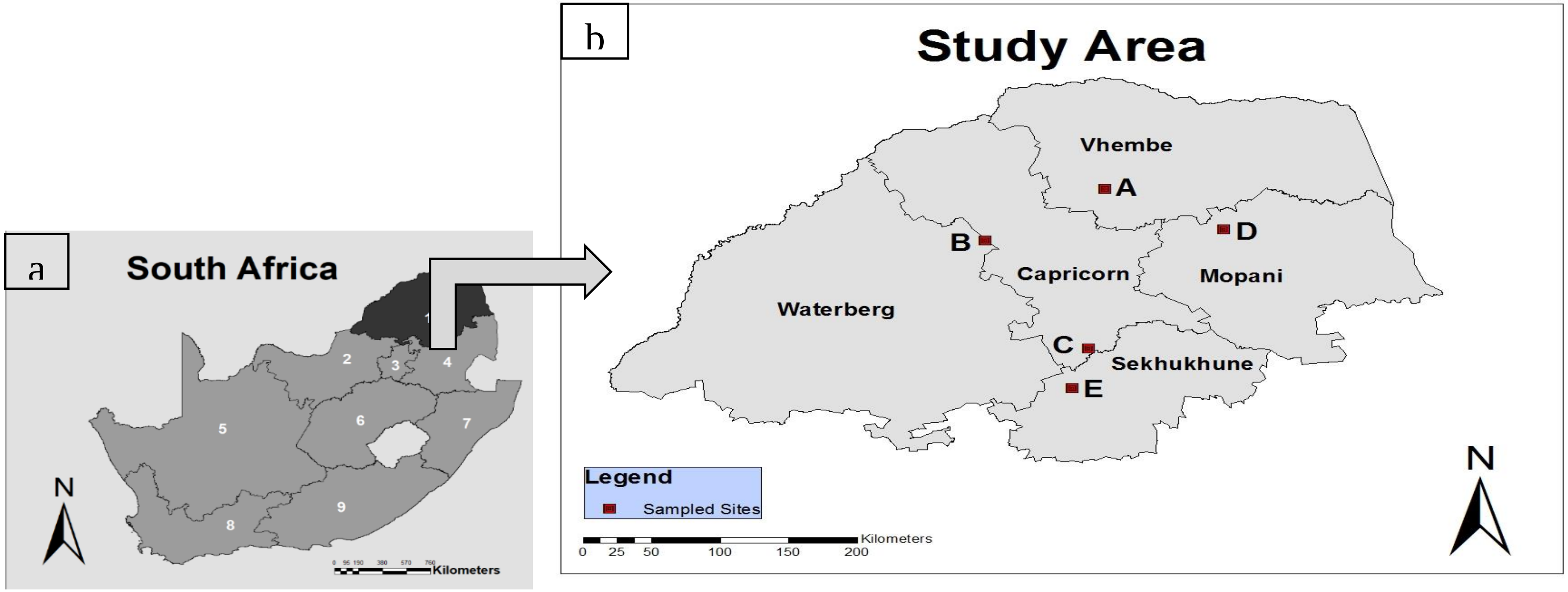

Figure 5.1: Maps showing study areas. (a) Map of South African provinces [1] Limpopo, [2] North West, [3] Gauteng, [4] Mpumalanga, [5] Northern Cape, [6] Free State, [7] KwaZulu-Natal, [8] Western Cape, [9] Eastern Cape. The study province (Limpopo) is shown in black colour. (b) Locality map of the sampled sites for anthelmintic resistance study from five districts of Limpopo province: (A) Madodonga village (Vhembe district), (B) Ga-Ramela village (Waterberg district), (C) Tooseng village (Capricorn district), (D) Mamokgadi village (Mopani district) and (E) Tompi Seleka College (Sekhukhune district). (Maps constructed using ArcGIS)

Farmers provided information regarding anthelmintic classes they use in their flocks, how often they treat their livestock, how often they change the active ingredients and whether or not they weigh their animals before treatment with anthelmintics. Consequently, experimental animals for FECRT were selected from the flocks that had a long history of anthelmintic treatment and high treatment frequencies in order to confirm development of AR. Five flocks with the highest treatment frequencies were selected from the 77 flocks with a total number of 650 sheep that were registered in the extension officer’s database. Faecal samples were collected from the animals that had not been treated for GI nematodes for at least eight weeks prior to sampling.

Faecal samples were collected during early summer in November and December 2017 for Capricorn and Sekhukhune districts whereas for Waterberg, Mopani and Vhembe, sampling was done during late summer in January and early autumn in February 2018.

5.3.3 *In vivo* assay: Faecal egg count reduction test (FECRT)

Individual counts of eggs per gram (EPG) for each sheep were determined by a modified McMaster technique with detection limit of 100 EPG from faecal samples (Reinecke 1983).

The FECRT was used to determine the presence of anthelmintic resistance as described by Coles et al. (2006). The minimum egg count for inclusion in the treatment groups, excluding the control group was 1,000 EPG.

All animals were weighed (plate 5.1) and ear tagged (plate 5.2) with different color codes specific to the anthelmintic groups and the controls.

Plate 5.1: Weighing sheep to determine the correct anthelmitic dose for FECRT

Ten animals per group were assigned to three different treatment groups and 10 animals for the control group to make 40 animals in each flock.

Plate 5.2: Eartagging of sheep in order to assign them to various treatment groups

Faecal cultures were prepared to identify nematode genera as described by Van Wyk et al. (2004) both pre and post-treatment. Group 1 was treated subcutaneously with ivermectin (Ivomec®, Merial, 0.2 mg/kg bw), group 2 was orally dosed with levamisole (Tramisol Ultra®, Coopers and Intervet, 5 mg/kg bw) and the third group was orally dosed with albendazole (Valbazen®, Pfizer, 7.5 mg/kg bw). Group 4 represented the untreated control.

5.3.4 In vitro assays

5.3.4.1 Egg Hatch Assay (EHA)

The egg hatch assay was conducted as described by Coles et al. (2006) and McGaw et al. (2007) for the determination of benzimidazole resistance *in vitro*. Nematode eggs were recovered from collected faecal samples using the nematode egg recovery method as described by Maphosa et al. (2010) with some minor modifications. Briefly, four grams of collected faecal sheep pellets were weighed, water was slowly added, and pellets were smashed until a relatively liquid suspension was obtained. The slurry was filtered through sieves of 117, 70 and 25 µm as shown below in figure 5.3 after which the contents of the 25 µm sieve were backwashed and transferred into a 50 ml centrifuge tube. The suspension was allowed to stand for 30 minutes and the sediments were suspended in a 40% sugar solution and allowed to stand for another 30 minutes. The supernatant was then washed through a 25 µm pore mesh sieve using distilled water. The nematode eggs were washed off from the 25 µm with distilled water, transferred back into the 50 mL tubes and allowed to stand for 2 hours. The concentrations of the eggs were estimated by repeatedly counting the number of eggs in 3 aliquots of 0.5 mL of the suspension in a microscope slide, after which the mean number of eggs per 0.5 mL was determined.

Plate 5.3: The sieves of 117, 70 and 25 µm used to recover nematode eggs from the sheep pellets

Approximately, 100 eggs of nematodes were pipetted into a 96 well microtitre plate and then 10 µl of thiabandezole (TBZ) solution added (Coles et al. 2006). The final concentrations of TBZ were 0.05, 0.1, 0.2, 0.3 and 0.5 µg/ml dissolved in dimethyl sulfoxide (DMSO). In addition, a negative control (distilled water) was tested. All tests were duplicated. The plates were covered and incubated for 48 hours at 27°C. A drop of Lugol's iodine solution was added to each well to stop further hatching and the number of unhatched eggs and the first stage larvae (L_1) present per well were counted. Inhibition percentages were calculated using a formula described by Cala et al. (2012).

$$\mathrm{E} = \frac{(\mathrm{Eggs} + \mathrm{L1}) - \mathrm{L1}}{\mathrm{Eggs} + \mathrm{L1}} \times 100$$

For the egg hatch assay (EHA), discriminating dose is a dose that prevents the hatching of 99% of susceptible eggs which means that the eggs that hatch are resistant (Coles et al. 2006). The level of resistance was determined by the number of eggs that hatched in the discriminating doses of 0.1 µg/ml TBZ.

5.3.4.2 Micro-Agar Larval Development Test (MALDT)

The MALDT was performed as described by Coles et al. (2006). The test was performed on 96 microtitre well plates. Stock solutions of TBZ and LEV were prepared by pre-dissolving the drugs in DMSO with subsequent dilution in distilled water (1:4). Nematode eggs recovered from faecal samples as described in the EHA above were incubated for seven days at 27ºC in 96-well microtitre plates. The plates had culture medium (yeast extract with Earle's Balanced Salt Solution and physiologic salt solution) in an aquatic solution of various concentrations (range from 0.0006 to 1.28 µg/mL) of TBZ and LEV (Coles et al. 2006) and the proportion of nematode eggs were determined for each well. The numbers of unhatched eggs and L_1 – L_3 larvae in each well were counted under an inverted microscope. The rate of L_3 development in the discriminating dose (0.02 µg/ml and 0.5 µg/ml for thiabendazole and levamisole, respectively) compared to the control was used to determine if resistance was present, thus the number of larvae developing from L_1 to L_3 stage in the discriminating dose of 0.02 µg/ml thiabendazole and 0.5 µg/ml levamisole was an indication of resistance. The test was performed in duplicates for each drug concentration.

5.3.5 Data analysis

The faecal egg count reduction percentage (FECR %) was calculated using the formula of Kochapakdee et al. 1995.

FECR%= 100 × [1− (T2/T1), where T2 represents FEC post treatment and T1 represent FEC pre-treatment (Kochapakdee et al. 1995).

Resistance was said to be present if the FECR% was less than 95 % and the lower limit of the 95% confidence interval was less than 90 %. If only one condition was met, then resistance was only suspected (Coles 1992; Vatta et al. 2001; Coles et al. 2006).

SAS Statistics (Version 9.4) was used to analyze FECRT data for overall confidence limits Instead of using the traditional threshold values (LC_{50} or LC_{99}), the threshold discriminating concentrations were used for both EHA and MALDT (Dolinska et al. 2013). Furthermore, the LC_{50} criterion is not able to provide early detection during the development of resistance (Dolinska et al. 2014). Microsoft® Excel 2016 and SAS Statistics (Version 9.4) statistical package were used to analyze questionnaire data.

5.4 Results

The questionnaire survey results indicated that all the farmers (100%) used visual appraisal of individual animal weight to estimate the anthelmintic dose used in sheep. High treatment frequencies of 3 times in a year for both flocks in Capricorn and Sekhukhune districts and 4 times in a year for flocks in Waterberg, Vhembe and Mopani districts were reported (Mphahlele et al. 2019). The frequency of change of anthelmintic with different mode of action ranged between never to every six months in the 5 districts (Figure 5.2).

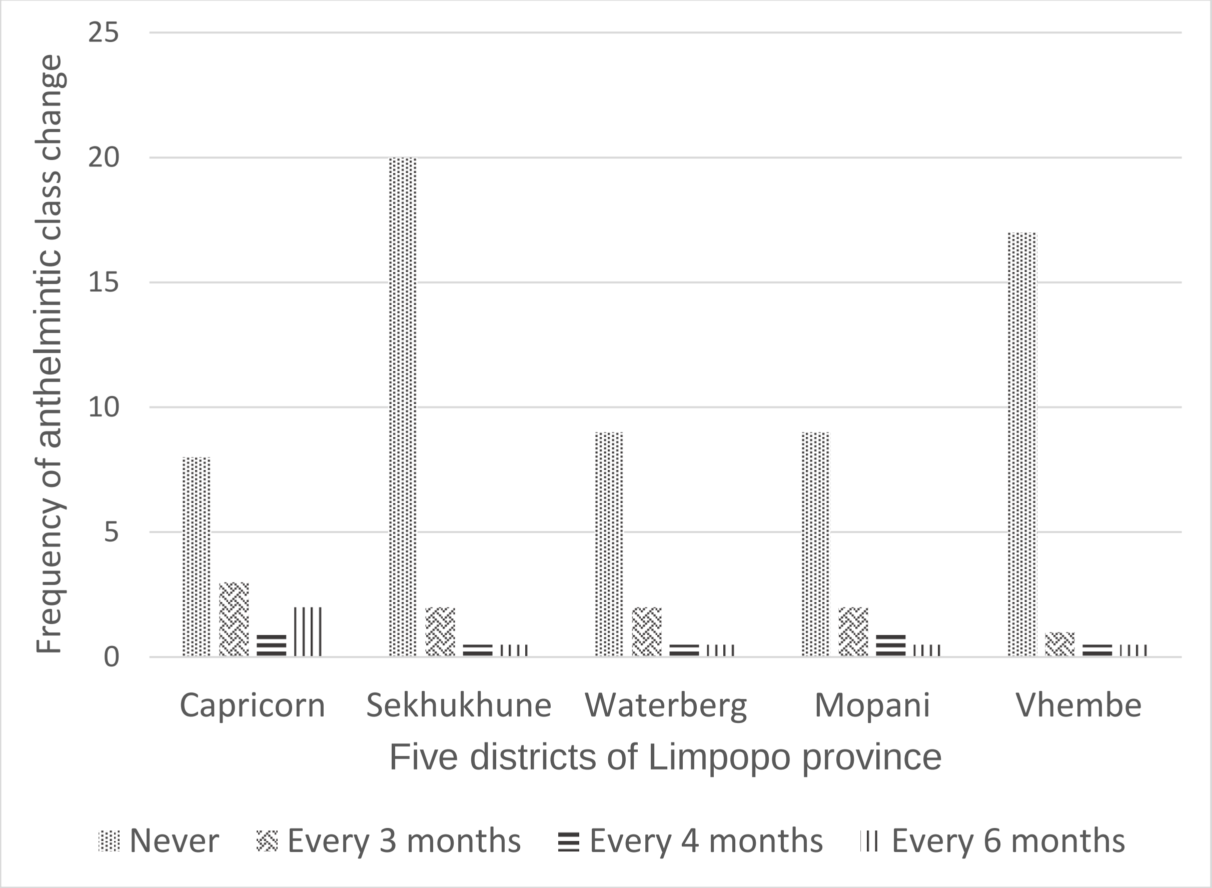

Figure 5.2: Frequency of anthelmintic class change in different districts of Limpopo province

Moreover, benzimidazole was the most used anthelmintic class in Sekhukhune and Vhembe districts with 95 and 94% usage respectively whereas the least used anthelmintic class was the macrocyclic lactone (Figure 5.3).

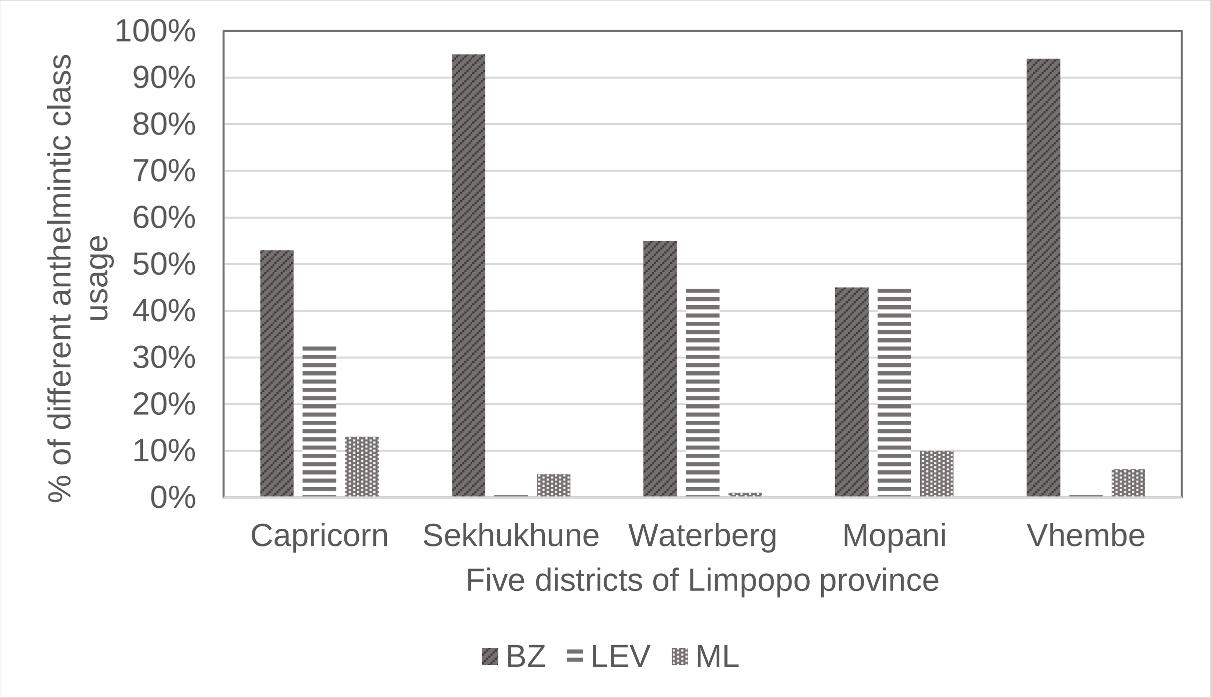

Figure 5.3: A trend of various anthelmintic class usage in different districts of Limpopo province

Using the analysis described by Kochapakdee et al (1995), the results revealed development of AR against all the anthelmintic classes with percentages of ≤ 95% and ≤ 90% lower confidence limit (LCL) in all the districts of Limpopo province except for Sekhukhune where AR was suspected against LEV and ML (Table 5.2). Furthermore, AR was also suspected against ML in Capricorn district flock at 92% FECR with LCL of 90%.

Table 5.2: Faecal egg count reductions and lower limits of 95% confidence level calculated on the basis of individual animal's egg counts before and after treatment on the same sheep using method of Kochapakdee et al. (1995). FECR% = 100 × [1− (T2/T1)

Study site	Anthelmintic used	FEC1 (Range)	FEC2 (Range)	FECR%	Lower limit of 95% confidence	Interpretation of results
Sekh	BZ	1940 (1455-2425)	80 (60-100)	96	93	Not resistant
	LEV	2080 (1560-2600)	150 (112-187)	94	90	Suspected resistance
	ML	2030 (1522-2537)	120 (90-150)	96	90	Suspected resistance
	Control	2080 (1560-2200)	2060 (1840-2560)	-	-	-
Capr	BZ	1790 (895-3580)	200 (100-400)	92	85	Resistant
	LEV	1660 (830-3320)	130 (65-260)	93	88	Resistant
	ML	1840 (920-3680)	120 (60-240)	92	90	Suspected resistance
	Control	1750 (1430-2080)	1940 (1850-2300)	-	-	-
W/berg	BZ	2840 (2200-3500)	1300 (400-2300)	56	40	Resistant
	LEV	2960 (1300-5000)	580 (200-900)	79	72	Resistant
	ML	4440 (2200-7200)	1340 (0-2000)	65	50	Resistant
	Control	1570 (1410-1600)	1580 (1380-1670)	-	-	-
Mopani	BZ	1960 (500-5000)	620 (300-880)	47	26	Resistant
	LEV	1620 (400-2300)	480 (100-900)	70	61	Resistant
	ML	1280 (500-2400)	320 (100-900)	72	51	Resistant
	Control	1180 (850-1250)	1180 (900-1250)	-	-	-
Vhembe	BZ	2660 (500-4400)	340 (0-1000)	89	82	Resistant
	LEV	2525 (400-7800)	50 (0-500)	90	78	Resistant
	ML	1060 (500-2000)	160 (0-300)	90	80	Resistant
	Control	1290 (1100-1350)	1280 (1080-1580)	-	-	-

Sekh = Sekhukhune; Capr = Capricorn; W/berg = Waterberg; BZ = Benzimidazole (Valbazen®); LEV = Levamisole (Tramisol Ultra®); ML = Macrocyclic Lactones (Ivomec®); FEC1= faecal egg count pre-treatment; FEC2= faecal egg count 14 days post-treatment

Faecal examination for the presence of nematode eggs in the pre-treatment faecal samples revealed that all sheep (100%) were positive for strongyle eggs. The three treatment groups had an average nematode egg count more than (>) 1,000 EPG pre-treatment whilst the control groups also had an average nematode egg count of >1,000 EPG. Nematode genera that were identified pre-treatment from the larval cultures included *Haemonchus contortus, Teladorsagia/Trichostrongylus* and *Oesophagostomum columbianum*. *Haemonchus contortus* was identified as the most frequently encountered resistant nematode post-treatment in all the five flocks. However, among all the nematodes genera identified pre-treatment, *Oesophagostomum columbianum* was the only species that was not detected post-treatment in all the anthelmintic treatments. (Table 5.3).

Table 5.3: Results of the percentage of gastrointestinal nematodes genera identified from the larval cultures at day 0 pre-treatment and day 14 post treatment

Anthelmintic group	***Haemonchus contortus.***		***Tel/Trich spp.***		***Oesophagostomum columbianum.***	
Sekhukhune	D 0	D 14	D 0	D 14	D 0	D 14
BZ	74	90	23	10	3	0
LEV	75	75	20	25	5	0
ML	87	96	10	4	3	0
Control	83	84	15	16	2	0
Capricorn						
BZ	93	97	4	3	3	0
LEV	87	93	10	7	3	0
ML	88	93	10	7	2	0
Control	82	86	15	14	3	0
Waterberg						
BZ	89	100	9	0	2	0
LEV	83	94	11	0	6	0
ML	85	100	10	0	5	0
Control	91	92	7	8	2	0
Mopani						
BZ	88	100	12	0	0	0
LEV	87	100	13	0	0	0
ML	95	100	5	0	0	0
Control	95	92	4	5	1	1
Vhembe						
BZ	90	100	8	0	2	0
LEV	90	100	5	0	5	0
ML	92	100	7	0	1	0
Control	93	92	6	4	1	2

Tel = Teladorsagia; Trich = Trichostrongylus; BZ = Benzimidazole; LEV= Levamisole; ML = Macrocytic Lactones

A total of 5 sheep flocks (1 flock in each of the 5 districts of Limpopo province) were investigated. The EHA results showed that TBZ, which is a benzimidazole showed resistance because it had a minimal ovicidal effect that resulted in more than 1% egg hatchability on nematode eggs at a discriminating dose (DD) of 0.1 µg/ml in all the 5 (100%) flocks that were investigated in Limpopo. Figure 5.4 shows the hatchability percentages at the DD of 0.1 µg/ml thiabendazole. The percentage of eggs hatching in the negative controls (water) for all flocks was >95%.

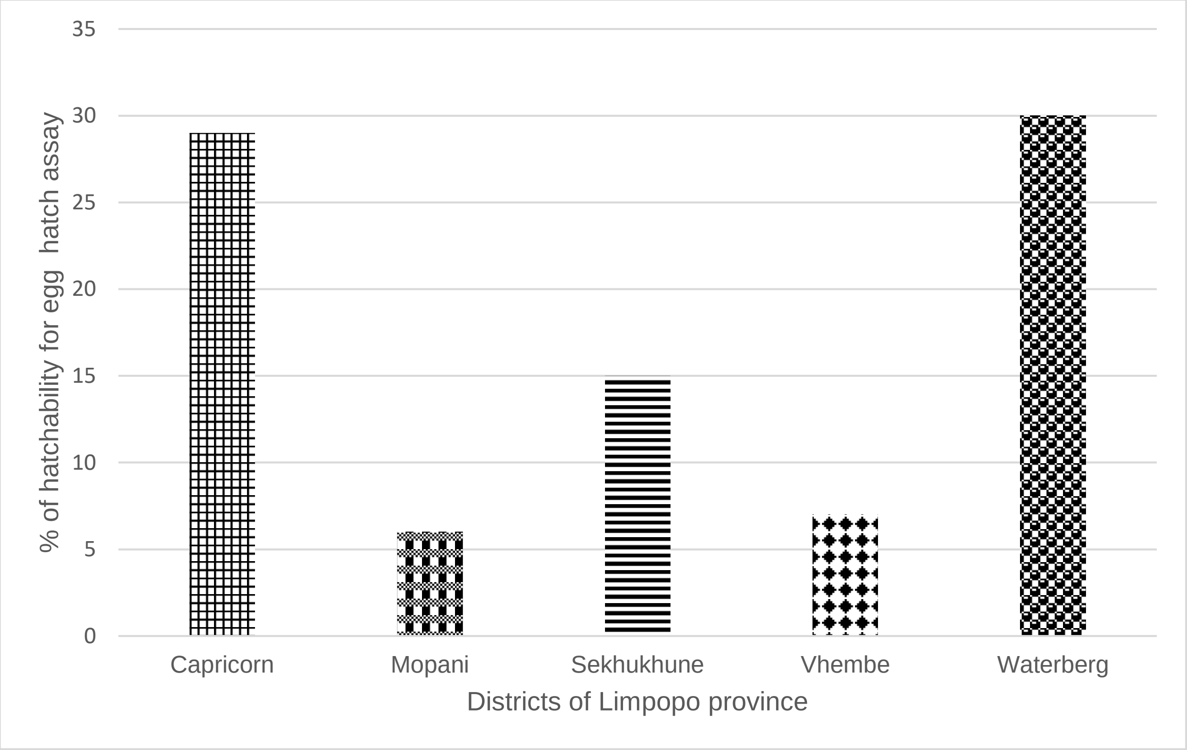

Figure 5.4: The percentage of eggs that hatched at a discriminating dose of 0.1 µg/ml thiabendazole

The MALDT showed no AR against LEV as 100% larval development inhibition was recorded in all the investigated flocks in Limpopo at 0.5 µg/ml DD. However, AR was detected against TBZ as development of the larvae from L_1 – L_3 was recorded in Sekhukhune, Capricorn and Waterberg flocks at 6, 22 and 29% respectively at 0.02 µg/ml DD (Figure 5.5).

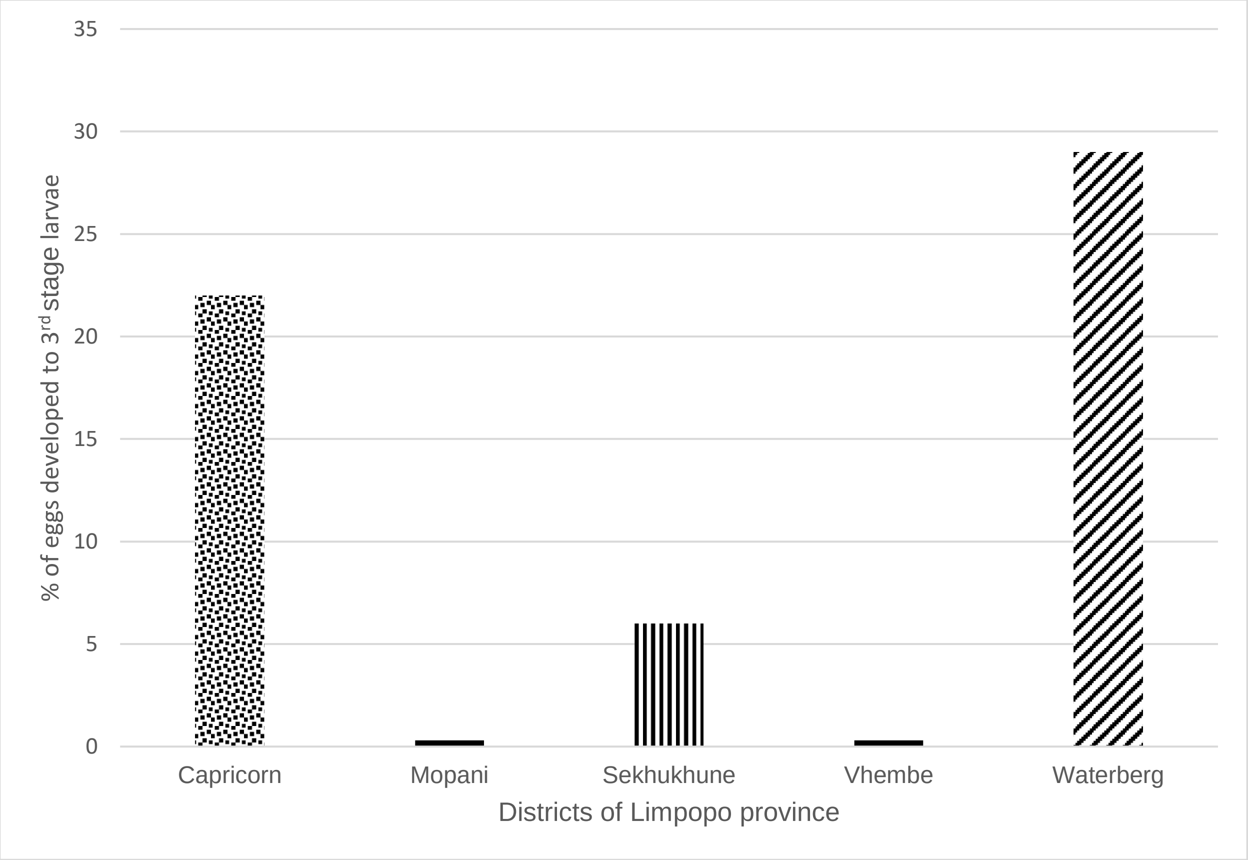

Figure 5.5: The percentage of eggs that developed to third stage (infective) larvae in the discriminating dose of thiabendazole (TBZ) (0.02 μg/ml) in the MALDT

5.5 Discussion

All the farmers (100%) who owned the flocks from which sheep for FECRT study were selected mentioned that they never weigh their animals before dosing them, but instead used weight estimates for determining the treatment dosages. In our previous study of risk factors leading to development of AR we also recorded this behaviour by small scale farmers in Limpopo province (Mphahlele et al. 2019). This is a practice that might well have led to under dosing which contributed to the detected AR for all three tested anthelmintic classes in this study, a view supported by the review of Vatta and Lindberg (2006). A recent study by Leathwick and Luo (2017) also evaluated the change in gene frequency at different dosing rates and concluded that AR was increased at a lower and more variable dose. AR has been demonstrated for most genera of GINs with varying drug types. Dosing with larger quantities and according to the manufacturer's recommendation worked efficiently with levamisole as the active principle (Baiak et al. 2018). Moreover, high treatment frequencies that were discovered in the questionnaire survey results could also be linked to AR that was observed both in the *in vitro* and *in vivo* studies and these findings correspond to the results of the studies conducted by Čerñanská et al. (2008) and Aga et al. (2013).

In our previous study of risk factors leading to development of AR we also recorded this behaviour by small scale farmers in Limpopo province (Mphahlele et al 2019). This is a practice that might well have led to under dosing which contributed to the detected AR for all three tested anthelmintic classes in this study, a view supported by the work of Vatta and Lindberg (2006). Furthermore, in the same study high treatment frequencies of 3 times in a year for both flocks in Capricorn and Sekhukhune districts and 4 times in a year for flocks in Waterberg, Vhembe and Mopani districts were reported. Eighty two percent of the resource-poor farmers in Limpopo province never change the anthelmintic class, 13% alternate the anthelmintic class every 3 months, 2.5% change them every four months and the remaining 2.5% change the anthelmintic class every 6 months. Moreover, benzimidazole was the most used anthelmintic class in Sekhukhune and Vhembe districts with 95 and 94% usage respectively, whereas the least used anthelmintic class was the macrocyclic lactone (Mphahlele et al. 2019).

Resistance was also confirmed by the hatching of eggs in the in vitro egg hatch assay (EHA) that is regarded as highly sensitive (Sherill et al. 2006).

In order to initiate discussion on the efficacy of the anthelmintics tested in the EHA study and to ultimately link it to the reduction in faecal egg counts and the helminth control practices employed in the sampled flocks, it is important to understand the concept of discriminating dose which is a dose that prevents the hatching of 99% of susceptible eggs as described by Coles et al. (2006). By definition, eggs that hatch are resistant. Present data suggests a dose of 0.1 µg/ ml thiabendazole will prevent the hatching of 99% of the parasite eggs. Studies conducted in the southern part of Ethiopia and Cuba established that benzimidazoles class of anthelmintics especially albendazole was the most commonly used by resource-poor farmers (Kumsa et al. 2010). In Limpopo province, an overwhelming majority of farmers ranging from 45% in Mopani to 95% in Sekhukhune districts use BZ to treat GINs of sheep and many of them never change the anthelmintic class. Low FECR% of 56% against BZ in Waterberg corresponded with the highest number of hatching eggs (30%) and the highest number of larvae that developed (29%) as compared to all the other 4 flocks. Nine out of 11 farmers (82%) in Waterberg never change the anthelmintic class, which is in this case BZ that was used in 55% of the sampled flocks. However, the same could not be said for macrocyclic lactones because the FECRT results showed that there is resistance against this anthelmintic class even though it was the least used in all the districts of Limpopo province, with no farmer in Sekhukhune and Waterberg ever mentioning its use in their flocks.

Earlier studies revealed that correlation between *in vivo* and *in vitro* tests is not always good (Dolinska et al. 2014). Maharshi et al. (2011) observed a poor correlation between FECRT results and EHA results for detecting BZ resistance. This is probably because *in vitro* tests are more sensitive than *in vivo* tests (Sherill et al. 2006). Even though some conflicting results were observed between the *in vitro* and *in vivo* assays in the present study, there are also outstanding cases where the results of both tests totally agree on flock resistance status. One such positive correlation is the non-development of larvae from L_1 to L_3 in the MALDT for detecting LEV resistance and high efficacies obtained from FECRT in both Sekhukhune and Vhembe district flocks even though the resistance status is only 'suspected resistance' because both the conditions that confirms resistance as described by Coles et al. (2006) (FECR of less than 95 % and a lower confidence interval of less than 90 %) could not be met. These results could be attributed to the fact that LEV is used rarely as compared to BZ in Sekhukhune and Vhembe district flocks as it is to the rest of South Africa and many European countries (Čerñanská et al. 2008). It is against this backdrop that with proper use that include administering the correct dosage for weight of the animal, pasture rotation and other management tools, anthelmintics can still be used with maximum benefits (Ketzis et al. 2006).

The FECRT results for both the FECR obtained in the present study for the three tested anthelmintic classes coupled with EHA and MALDT results indicates development of resistance in all the flocks except for Sekhukhune district flock where AR was suspected against LEV and BZ. Another exception was for Vhembe district flock where AR was only suspected against LEV. These results compare favourably with those of earlier work in Limpopo province almost two decades earlier that revealed levamisole was < 95% effective and benzimidazole, only between 75-85% effective (Van Wyk et al. 1999). The predominance of the genus *Haemonchus* both pre and post treatment in the sheep belonging to small scale farmers in the five districts of Limpopo is consistent with the results obtained from studies conducted in sheep raised under small-scale resource-poor farming conditions in other areas of South Africa namely, Limpopo province (Van Wyk et al. 1999); North West province (Bakunzi 2003); Gauteng (Tsotetsi et al. 2013) where the most prevalent genus was *Haemonchus*. The reduction of *Trichostrongylus/Teledorsagia* spp. and *Oesophagostomum columbianum*. and increase of *Haemonchus contortus* post-treatment could be attributed to unrestricted growth potential of the population and shorter generation interval of *Haemonchus* spp. which allows for greater contamination of pastures and re-infection of animals (Torres-Acosta et al. 2003). In addition to unrestricted growth potential of the population and shorter generation interval, a mature female of *Haemonchus* spp. can produce in excess of 5000 eggs per day while *Trichostrongylus* spp. produce only up to 200 eggs per day which shows a great difference in their reproduction abilities (Getachew et al. 2007). On the other hand, *Oesophagostomum* sp. has a very long generation interval of 45 days that causes its prevalence to be much lower (Wahab and Adanan 1992).

In their earlier study Van Wyk et al. (1999) reported that more than 90 % of the farms harboured *Haemonchus* strains that were resistant to at least one of the four anthelmintics tested and that between 60 and 78 % of these strains were resistant to three anthelmintic groups.

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CHAPTER 6: GENETIC DIVERSITY OF *HAEMONCHUS CONTORTUS* IN LIMPOPO PROVINCE, SOUTH AFRICA: IMPLICATIONS FOR SPREAD OF ANTHELMINTIC RESISTANT STRAINS

Abstract

The current study was aimed at determining the genetic diversity as well as the phylogenetic relationship of *Haemonchus contortus* isolates from naturally infected sheep in Limpopo Province, South Africa, with isolates elsewhere, using the ITS2 DNA region. Sequence divergence ranged from 0.000 to 0.045, with a mean of 0.015. The nucleotide diversity (π) was 0.015, while haplotype diversity (*h*) was 0.690. However, no polymorphism was observed within the Limpopo *H. contortus* isolates. Phylogenetic analyses revealed four major lineages where Limpopo isolates shared common ancestry with reference sequences from Africa, as well as across other continents. The only isolatess that did not cluster with the South African isolates were isolates from the USA. Low levels of structuring observed in the present study and among other isolates elsewhere could imply a high level of gene flow due to population mixing across regions. The findings of this study may provide a basis for future studies in understanding and controlling the spread of anthelmintic resistant strains of *H. contortus* in Limpopo province and South Africa at large and also for tracing changes in the population structure of *Haemonchus contortus* in Limpopo Province.

Key words: *Haemonchus contortus*; nucleotide diversity; ITS2; haplotype diversity; gene flow; anthelmintic resistance; Limpopo Province

6.1 Introduction

Haemonchus contortus infection is great concern among trichostrongylid nematodes of small ruminants worldwide (O'Connor et al. 2006). It inhabits the abomasum and has a high biotic potential with the females laying 5000–10,000 eggs per day (Abutarbush 2010). It causes blood loss (0.05 ml/parasite/day) leading to serious health effects such as anemia, edema (bottle jaw) and even death in severely affected animals (Taylor et al. 2007). The parasite is responsible for serious production and economic loss in terms of reduced body weight, cost and labor for anthelmintic treatment and mortality of animals. The estimated economic loss by this parasitic infection is $26 million, $46 million and $103 million per annum for Kenya, South Africa and India, respectively (Gasser et al. 2008). In addition, *H. contortus* in goats and sheep is able to develop multiple resistance to many classes of anthelminthic drugs such as benzimidazoles, macrocyclic lactones, levamisole and imidazothiazoles (Papadopoulos et al. 2012). Generally, control of parasitic nematodes in livestock focuses on the use of anthelmintic drugs as well as farm management practices (Gasser et al. 2008). However, the knowledge derived from genetic information regarding this parasite such as genetic diversity and phylogenetic status is necessary to increase understanding of the ecology, epidemiology and evolution of parasitic nematodes (Jacquiet et al. 1995), and also to increase the efficacy of control programs. The population genetic structure of the *H. contortus* is promoted by a high rate of gene flow among populations (Yin et al. 2013).

Nematodes like *H. contortus* possess high levels of prolificacy coupled with a high rate of infection and a life cycle without an intermediate host, thus leading to a large effective population size with wide genetic variability (Prichard 2001). The high degree of intra-population diversity is an important characteristic of the trichostrongylids, especially *Haemonchus* species (Blouin et al. 1998). Silvestre et al. (2009) also reported that the diversified genetic features of this parasite have an impact on the development and spread of anthelmintic resistance (AR). Taxonomic and phylogenetic studies are essential to achieve a better understanding of the ecology, epidemiology and evolution processes of parasitic nematodes (Cabaret 2003; Mejı´a-Madrid et al. 2007). Population genetic studies of *H. contortus* have been conducted in a wide range of geographical regions of the world, including Australia, Brazil, Europe, Malaysia, and the USA (Troell et al. 2006). However, surprisingly, nothing is known about genetic variability within *H. contortus* populations in South Africa, in spite of its endemic status and economic impact in the country. Therefore, this study explored genetic diversity among ten populations of *H. contortus* from Capricorn, Sekhukhune, Waterberg, Mopani and Vhembe districts of Limpopo province, South Africa, using the ITS2 gene as a marker.

6.2 Materials and methods

6.2.1 Collection of faecal samples for culture and extraction of DNA from *Haemonchus contortus* larvae

Fresh faecal samples were directly collected from the recta of sheep in all five districts of the Limpopo Province, namely: Sekhukhune, Capricorn, Waterberg, Mopani and Vhembe (Refer to figure 5.1) and stored in a 4°C cooler box until subsequent analyses were conducted. Nematode egg counts from two sheep flocks per district were determined using the modified McMaster technique (Reinecke 1983). Faecal cultures were prepared according to Reinecke (1983) and the resulting third stage nematode larvae were identified to the genus level as described by Van Wyk et al. (2004). Chelex® method (Straube and Juen 2013) was used for DNA extraction. Briefly, 30 µl of Chelex® was added to the DNA vial containing approximately 100 larvae of gastrointestinal nematodes of sheep and then 5 µl of Proteinase K was added to the samples and vortexed briefly before being centrifuged at a maximum speed of 5000 rpm for 1 minute. All DNA samples were stored in a -20°C freezer for future downstream analysis. Appendix 2 provides a sample list of *H. contortus* individuals isolated from sheep in the five districts in Limpopo Province, South Africa.

The PCR targeting ITS2 gene was conducted to amplify 259 DNA fragment using species-specific primers (HAE-F CAA ATG GCA TTT GTC TTT TAG and NCR-2 TTA GT TTC TTT TCC TCC GCT) (Bott et al. 2009) under the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 15 s and extension at 72°C for 45 seconds, with a final elongation step at 72°C for 7 min. Amplified products were electrophoresed on a 1% agarose gel stained with 1 µg/ml ethidium bromide and visualised under UV light. All positively amplified products were sent for sequencing using the same primers at Inqaba Biotech, Pretoria (South Africa). For more precision, sequencing of amplicons was done in both forward and reverse directions.

6.2.2 Data analysis

Generated sequences in AB1 format were manually edited using Trace Editor on MEGA 7 (Kumar et al. 2016). Thereafter, all sequences in FASTA format were subjected to nucleotide BLAST (Boratyn et al. 2013) on the NCBI database (Benson et al. 2013) for DNA region homology confirmation. Additional sequences for other *H. contortus* from other parts of the world were downloaded from the NCBI database to infer the relationship of the Limpopo isolates from this study to other localities. The accession numbers (acc. no.) of the downloaded sequences were HQ844231, MG669349, MF398432, JN128898, KC998714, KJ724297, KU870651, KF176320, EU084688, EU086385, EU084689, EU084687, EU086382, EU086379, EU086377, EU084690 and EU086389. Split ends on the alignment were trimmed for uniformity. The sequences were then aligned using the Clustal W plugin (Thompson et al. 1994) and subsequently saved as FASTA format.

For phylogenetic analysis, two methods were employed, namely, neighbour-joining and maximum likelihood methods. Best substitution models estimate analysis was done using the model test plugin on MEGA 7 and the Tamura-3 parameter model (T92+G) was used as the best model for our dataset (Nei and Kumar 2000; Kumar et al. 2016). Neighbour-joining and maximum likelihood trees were constructed on MEGA 7. For maximum likelihood, 1000 bootstrap support replicates and a discrete Gamma distribution for evolutionary rate differences among sites was set at 5 categories. For neighbour-joining tree, the bootstrap analysis was performed by implementing 1000 replicates. For both methods of analysis, *Haemonchus similis* (acc. no. KY741876 and KY741879) was used as an outgroup. Haplotype networks were inferred by implementing the median-joining method in Popart (Leigh and Bryant 2015), using default parameters. Molecular diversity indices were estimated in DnaSP (Rozas et al. 2017) and MEGA 7 (Kumar et al. 2016). These included the overall mean and between sequence divergence, nucleotide diversity and gene diversity. Neutrality test was performed by estimating the Tajima’s D parameter in DnaSP.

6.3 Results

The ITS2 gene sequences were successfully generated from nine of the ten PCR positive samples. One PCR product failed a quality check and was subsequently excluded from the analyses. The sequence information is summarized in Table 6.1. All the sequences obtained in this study were submitted to GenBank under the accession numbers

MN299318; MN299319; MN299320; MN299321; MN299322; MN299323; MN299324; MN299325 and MN299326.

Table 6.1: Summary of the sequence information obtained from *Haemonchus contortus* from Limpopo, South Africa and five other continents inferred from the ITS2 DNA region

	ITS2 (without outgroup)
Model of evolution	T92
Length	166
R	1
Conserved	149 (152)
Variable	16 (11)
Singletons	6 (6)
Parsimonious-informative	10 (5)
Thiamine	33.9 (33.7)
Cytosine	16.0 (16.2)
Adenine	28.5 (28.5)
Guanine	21.5 (21.6)

The alignment length was 166 bp after trimming, and included 27 *H. contortus*, 15 of which were reference sequences from GenBank. Hundred and fifty-two of the sites were conserved, eleven were variable, of which six were singletons. Five of the sites were parsimoniously-informative. The base composition was 33.7% T, 16.2% C, 28.5% A and 21.6% G. Molecular diversity parameters are summarized in Table 6.2 below. Briefly, the overall mean sequence divergence was 0.015 (0.005). The minimum pairwise sequence divergence was 0.000, while the maximum was 0.045 (Table 6.2). The individual pairwise sequence information is available in appendix 3. The nucleotide diversity (π) was 0.015, while haplotype diversity (h) was 0.690 (Table 6.2). However, no polymorphism was observed within the Limpopo *H. contortus* isolates.

Table 6.2: Summary of the molecular diversity in *Haemonchus contortus* from Limpopo province, South Africa and five other continents inferred from the ITS2 DNA region

n	**Haplotype diversity (Hd)**	**Nucleotide diversity (π)**	**Overall mean distance(d) (S.E)**	**Pairwise distance range**	**Tajima's D (*P-value*)**
27	0.690	0.015	0.015 (0.005)	0.000-0.045	-0.788 (P>0.10)

The phylogenetic trees inferred using both the maximum likelihood and neighbour-joining methods produced similar topologies (Figure 6.1). There were four major branches observed in both trees. However, the clades were not well-resolved and the bootstrap support values were low (Figure 6.1).

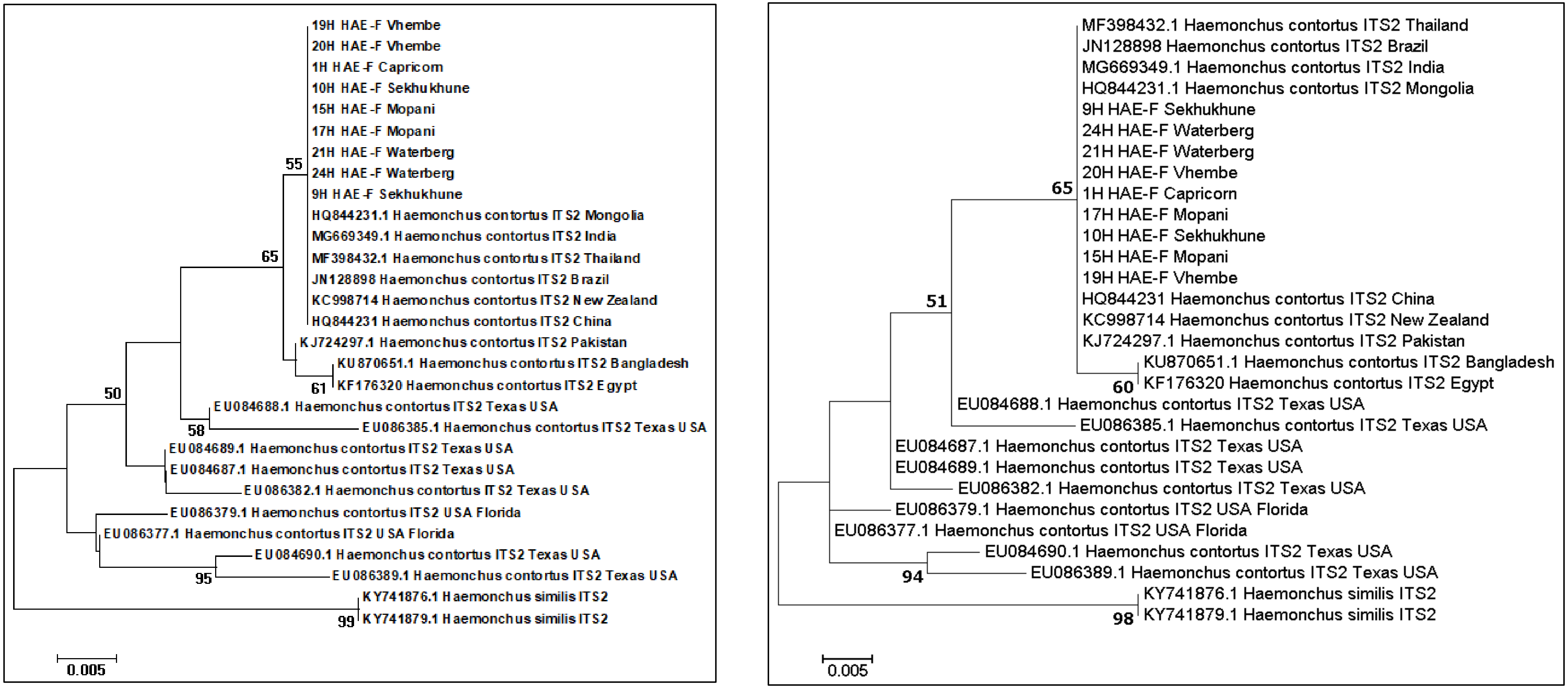

Figure 6.1: Phylogenetic relationship of *Haemonchus contortus* isolates from Limpopo, South Africa and five other continents based on the ITS2 DNA region. The trees were inferred using the (a) neighbour-joining method and (b) maximum likelihood method. Bootstrap support values are indicated on the branches

Nevertheless, the clade with the Limpopo samples was well-resolved. Limpopo isolates clustered with reference sequences from Africa (Egypt), as well as across other continents (Asia, North America, Oceania, and South America). The only individuals that did not cluster with the South African isolates were from the USA, where multiple haplotypes were observed.

Haplotype analysis was performed as no heterozygous alleles were observed in most of the sequences in the dataset – the only observed heterozygote (acc. no. KJ724297.1) was removed from the analyses. Seven *H. contortus* haplotypes were observed in this study (Figure 6.2).

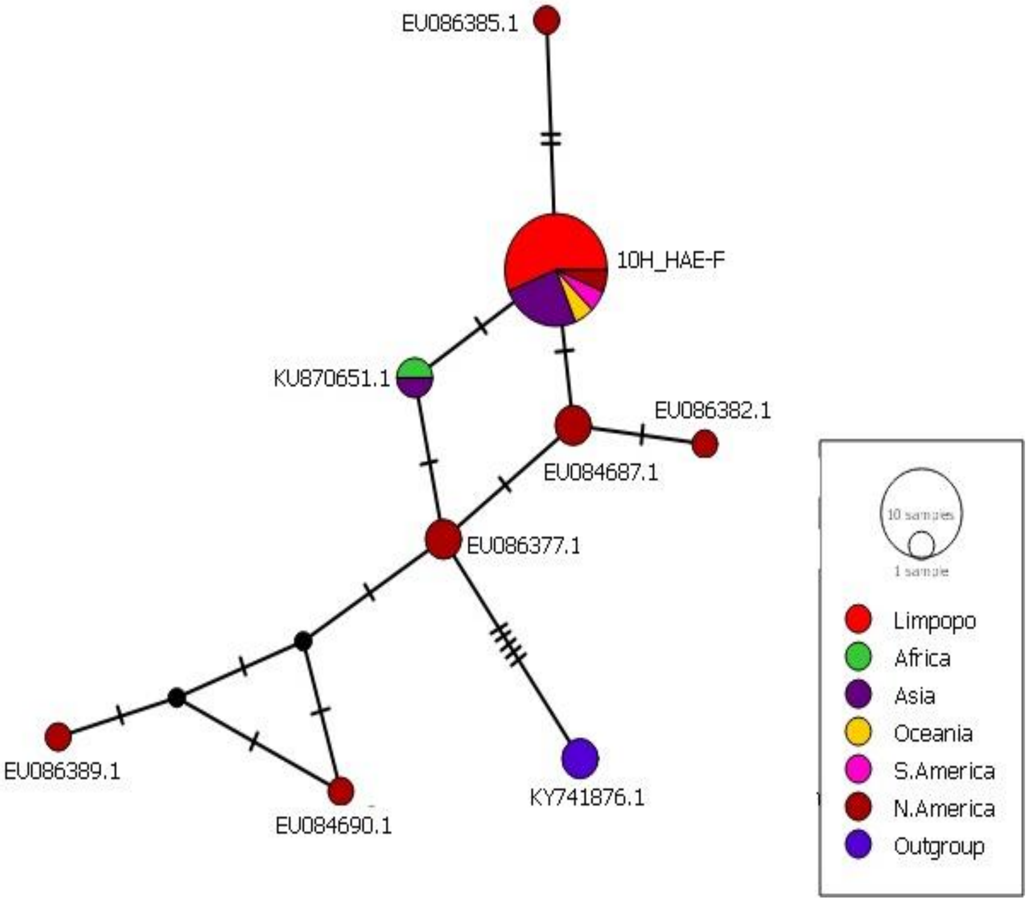

Figure 6.2: Haplotype network of *Haemonchus contortus* from Limpopo, South Africa and five other continents based on the ITS2 DNA region. The lines across branches indicate mutational steps. The black circles represent unsampled haplotypes

One out of the seven haplotypes shared between Limpopo Province isolates and isolates from four continents, one haplotype was shared between an African and an

Asian isolates, while the remaining five haplotypes were found in the USA only. The Afro-Asiatic haplotype diverged from the South African haplotype by one mutational step. One of the USA haplotypes diverged from the South African haplotype by one mutational steps, while three diverged by two mutational steps. The other two USA haplotypes were separated from the South African haplotype by five mutations, as well as one and two missing haplotypes, respectively. Tajima's D was negative but not significantly different from a neutrally-evolving population or DNA region (-0.788; P>0.10).

6.4 Discussion

H. contortus is a parasite of high biotic potential, together with high infection rate in small ruminants and a direct life cycle which results in a large effective population with marked genetic variability (Blouin et al. 1998; Prichard 2001). Lack of nucleotide diversity that was recorded for all the five districts of Limpopo Province and between Limpopo populations and Brazil, China, India, Mongolia, Thailand, New Zealand and Pakistan populations suggests that a low genetic differentiation and a high gene flow exist between the *H. contortus* populations in the Limpopo Province and across the world. The pairwise estimation of evolutionary divergence results between sequences in the present study revealed that there was no genetic diversity between *H. contortus* populations of Limpopo province in South Africa and the rest of the world.

The distinct clustering of the USA populations suggests that they do not have a common ancestry with populations from the rest of the world. However, one isolate from the USA shared the same haplotype as Limpopo individuals, as well as one isolate with only one mutational differentiation from Limpopo. The multiple branches that were observed in the present study suggests that the USA has the highest diversity. An earlier study from USA has shown that *H. contortus* exhibits high population variation and low genetic differentiation within continuous geographical regions, likely ascribing to high gene flow influenced by host movement (Anderson et al. 1998). However, strong barriers to gene flow have also been observed on a global scale, which appear to be attributed to poor dispersal ability of the parasite and restricted opportunities for host movement across continents (Troell et al. 2006). In their studies, Troell et al. (2006) and Yin et al. (2013) have observed a modest level of divergence (genetic distance of < 0.1) in *H. contortus* populations within continents although there is a more marked divergence globally (genetic distance of > 0.1). Low levels of structure could imply a high level of gene flow that could be the result of population mixing (migration) across regions. This study tends to point out that *H. contortus* populations are not being limited geographically despite control efforts. The minimum pairwise sequence divergence in the present study indicates the presence of divergent lineages. The mean nucleotide diversity of 0.015 and the mean haplotype diversity of 0.690 for the 27 individuals of *H. contortus* in this study implied an excess of low frequency polymorphism. On the other hand, a conserved sequence and a negative Tajima's D could also indicate levels purifying selection, a selective sweep or population growth (Tajima 1989; Fu 1997). However, Tajima's D was not significantly different from a region undergoing selection, albeit being negative. Thus, it is likely that the lack of genetic differentiation between the Limpopo populations and the other regions is a result of high gene flow facilitated by host movement across continents. The lack of diversity could also indicate that the ITS2 region is conserved among Limpopo populations, and that the genotype observed in Limpopo and the other regions originated from a common ancestor. The high sequence divergence among populations within the same geographical regions (e.g USA) and low divergence between different regions (as observed between Limpopo versus Asia, South America, etc) in the present study compares well with the observations of Yin et al. (2013) where nucleotide diversities ranged from 0.0178 to 0.0369, and the haplotype diversity ranged from 0.993 to 1.000 in a China study. In the present study, there was higher sequence divergence among individuals from the same continents (North America, Asia and Africa) than between continents (same haplotype found in all five continents). It is important to note that the high level of genetic similarity between the Limpopo isolates could also be due to other factors other than just gene flow; factors such as over-lapping selection pressures and common ancestry effects between populations (Malatji et al 2016).

Genetic diversity in nematodes leads to variation in response to anthelmintics (Prichard 2001). *Haemonchus contortus* shows enormous genetic diversity, allowing anthelmintic resistance alleles to be rapidly selected (Prichard 2001). Anthelminthic resistance is now a widespread problem, especially in *H. contortus*, and South Africa is no exception. Alarmingly, there is no registered vaccine against haemonchosis in South Africa and just like elsewhere in the world, the control of *H. contortus* relies largely on anthelmintics. The change in single nucleotide from tyrosine to alanine selects an individual for resistance (Irum et al. 2014). This mutation is also responsible for BZ resistance in other nematodes species including *Caenorhabditis elegans*, *Cylicocyclus nassatus* and *Cyatostomum coronatum* (von-Samson-Himmelstjerna et al. 2001). However, the region analysed in this study is a non-coding region. While non-coding regions may not directly indicate resistance, association of genotypes with resistant phenotypes may help identify and track the movement of resistant individuals across regions and populations (Chen and Tian 2016). The Limpopo lineage in this study shared the same genotypes as AR individuals from North America (Garretson et al. 2009). Thus, the high level of gene flow observed in this study indicates that AR genotypes may be easily spread worldwide, and that host movement and treatment practices are crucial to the management of *H. contortus* worldwide.

In our previous study that investigated the risk factors associated with AR in Limpopo province, we established that none of the resource-poor farmers in Limpopo weighed their sheep before dosing but relied solely on the visual appraisal of an animal to determine its weight which may be a cause of under-dosing, and consequently, resistance to anthelmintics may follow in Limpopo province (Mphahlele et al. 2019). In that particular study, it was also established that high treatment frequency was another risk factor where farmers who treat their flocks two or more times per year do so without coprological assessment for the necessity of treatment or its efficacy afterwards and that puts the flocks at risk of AR (Mphahlele et al. 2019). Those results could well be linked to the mutation observed in the present study.

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CHAPTER 7: CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

This study has updated the occurrence of gastrointestinal parasites infecting sheep in all districts of Limpopo province which has appeared to be higher during hot wet season and the infections were directly related to anemia as determined using FAMACHA© scores. Results of the composite faecal cultures revealed four species of nematodes in all the five districts of Limpopo province, namely, *Haemonchus contortus, Trichostrongylus/Teladorsagia spp* and *Oesophagostomum columbianum*. *Haemonchus contortus* were the dominant species, representing a bigger part of the total larval recovery throughout the entire study period in all the districts of Limpopo province followed by *Trichostrongylus/Teladorsagia* spp. and *Oesophagostomum columbianum* which was detected in small percentages. Other common species such as *Cooperia* were not recorded in this study. This could be because of dominant species such as *haemonchus* suppressing them. The high prevalence of GINs in Limpopo province could be due to conducive climatic conditions that support prolonged survival of infective larvae such as high temperatures and rainfall that prevailed in the province during the study period. The positive correlation between faecal egg count and clinical anemia severity suggests that worm burden was the main contributory factor to anemia.

The study further documented risk factors associated with development of AR. Important observations and opinions emerged that reflect serious constraints for resource-poor farmers in Limpopo province of South Africa. Among other things, present study has shown that the anthelmintic drenching practices such as incorrect dosages used in sheep flocks of resource poor farmers in Limpopo province caused by administration of anthelmintics without weighing, may contribute to the development of AR. None of the sheep flocks were weighed for calculating correct anthelmintic dose and that represents a high risk for under-dosing anthelmintics in these flocks. Therefore, AR observed in sheep in this study may be due to under dosing as a result of lack of weighing equipment and high treatment frequencies due to lack of proper training on anthelmintic use in Limpopo province.

The *in vivo* and *in vitro* detected the presence of AR in most districts for ivermectin and albendazole. In addition, resource poor farmers often buy small stock at auctions supplied by commercial farms on which AR is common and they also move sheep between districts which may lead to resistant worm strains being disseminated. This occurrence of AR in the resource poor-small stock farming sector is cause for concern.

Genetic analysis of the ITS2 gene of *H. contortus* revealed that there is no genetic variability amongst Limpopo isolates and that they cluster with isolates from other countries except USA. These findings provide a snapshot of the genetic make-up of *H. contortus* populations in Limpopo Province, South Africa, and to the best of our knowledge, this is the first study on genetic variability of *H. contortus* infecting sheep in Limpopo province and in South Africa. Results from the study revealed a low population genetic differentiation and high gene flow of *H. contortus* in Limpopo province. On a global scale, there were low levels of differentiation among different continents, although there was an observed level of divergence within same continents where multiple individuals could be sourced (Africa, Asia and North America). The findings of the present study may provide a basis for future studies and it can also be used to trace changes in the population structure of *H. contortus* in Limpopo province and the rest of South Africa.

The present study has shown that the resource-poor farmers in Limpopo province have limited knowledge on the correct use of anthelmintics and that has a direct negetive impact on the development of AR and the potential spread of resistant strains. This has been demonstrated by the conducting a genetic analysis of the ITS2 gene of *H. contortus* that revealed there is no genetic variability amongst Limpopo isolates. Furthermore, the seasonal prevalence data of GINs and the associated aenemia that increases during the hot-wet part of the year provides insights into the disease epidemiology of haemonchosis. The data generated in this study will contribute to re-formulation of control efforts of GINs infecting sheep in Limpopo by encouraging resource-poor farmers to use new methods such as targeted drenching to prevent AR development.

7.2 Recommendations

- In order to avoid or slow down the emergence of AR, correct use of anthelmintics and on-farm training about gastrointestinal nematodes infecting small stock must be provided. Such training should be ongoing and provided by extension officers together with animal health technicians. Training initiatives should incorporate practical demonstrations and focus on aspects such the importance of correct dosage, when to alternate anthelmintic classes, treatment frequency and new treatment strategies, such as targeted drenching in combination with faecal egg counts. It is also suggested that government veterinary services at district and municipality levels purchase the weighing bands which animal health technicians can always carry along during branding, vaccination and dipping campaigns so that they can also assist farmers with weight determination in order to ensure they can administer accurate doses of anthelmintics.
- The FAMACHA© system may be used to correctly mark those animals which require anthelmintic treatment and consequently slow down the development of anthelmintic resistance.
- As the level of gene flow among populations of *H. contortus* infecting sheep in Limpopo province was shown to be high, there is an opportunity for resistance alleles to spread, warranting an urgent need to investigate drug resistance in *H. contortus* throughout South Africa to assess the prevalence and intensity of anthelmintic resistance.
- Similar studies on AR development risks, *in vivo* and *in vitro* AR assessments must be conducted in the rest of SA provinces where sheep and goat farming is common in order to give a broader picture of GIN infections and AR development.

Appendixes

Appendix 1: Risk factors associated with the occurrence of anthelmintic resistance in Limpopo province, South Africa

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Questionnaire no	

Name of interviewer	
Name of translator (if any)	
Village	
Ward no.	
District	
Municipality	
Locality co-ordinates	
Province	
Date of interview	

Dear Sir/Madam

We would be honoured if you could assist us with this questionnaire to help us find out about the use of medicinal plants to cure livestock from internal parasites in this area. Your personal information will be kept confidential. The results of our study will assist with the formulation of strategies to control internal parasites and better use of anthelmintics. Thanking you in advance for your help.

Name and Surname:						
Address:						
Cell phone number:						
Gender	Male		Female			
Age group	21 - 30		31 - 40		41 +	

1. How long have you been farming sheep?

Less than 5 years		From 6 to10 years		More than 10 years	

2. How many sheep do you have?

Less than 40		between 40 to 80		More than 80	

3. What is your level of education?

Below matric	Post matric

4. Which other animal species do you keep, and how many are they?

Species	**Number of animals**
Cattle	
Donkeys	
Goats	
Pigs	
Poultry	
Total	

5. Are you aware of internal worms that infect livestock?

yes	
No	

6. Do you know how animals become infected with internal parasites? If yes, please elaborate.

yes	
No	

7. Can you identity an animal that infected with worms? If yes, please explain.

yes	
No	

8. Do you have animals dying from internal parasites in your flock? If yes, how many?

yes	
No	

9. Can you identify an animal that was killed by internal parasites?

yes	
No	

10. Do you know which months of the year do the animals show the signs of worm infection? If yes, please mention them.

yes	
No	

11. Do you dose your animals with commercial drugs? If yes please specify.

yes	
No	

12. Do you ever change the brand name that you use? If yes how often and why?

yes	
No	

13. Do you ever use alternative medicine to treat your animals against internal parasite infestations? If yes what do you use?

yes	
No	

14. Do you know how much medicine you must give to your animals?

yes	
No	

15. Do you weigh your animals before dosing? If yes, why?

yes	
No	

16. Do you know if the remedies are working/effective? If yes how do you know?

yes	
No	

17. How often do you administer the medicine to your livestock? If other, please specify

Once a year		Twice a year		When necessary		Other	

18. Any other comments on the usage of anthelmintics to treat internal parasites in livestock?

Appendix 2: Sample list of *Haemonchus contortus* individuals isolated from sheep in the five districts in Limpopo Province, South Africa

Sample	**District where it was isolated**
1H HAE F Capricorn	Capricorn
2H HAE F Capricorn	Capricorn
9H HAE F Sekhukhune	Sekhukhune
10H HAE F Sekhukhune	Sekhukhune
15H HAE F Mopani	Mopani
17H HAE F Mopani	Mopani
19H HAE F Vhembe	Vhembe
20H HAE F Vhembe	Vhembe
21H HAE F Waterberg	Waterberg
24H HAE F Waterberg	Waterberg

Appendix 3: Pairwise genetic distance between *Haemonchus contortus* isolates inferred from the ITS2 DNA region

	10H	KU870651	KJ724297	EU084688	EU084687	EU086382	EU086377	EU084690	EU086389	EU086379	EU086385	KY741876 HS
10H HAE-F***		0.006	0.000	0.009	0.011	0.013	0.012	0.015	0.016	0.014	0.013	0.020
KU870651	0.006		0.000	0.011	0.012	0.014	0.011	0.014	0.015	0.013	0.014	0.013
KJ724297**	0.000	0.000		0.009	0.009	0.012	0.009	0.013	0.014	0.011	0.013	0.011
EU084688	0.012	0.019	0.013		0.006	0.009	0.008	0.012	0.014	0.010	0.009	0.020
EU084687*	0.019	0.025	0.013	0.006		0.006	0.006	0.010	0.012	0.008	0.011	0.018
EU086382	0.025	0.032	0.019	0.012	0.006		0.009	0.012	0.014	0.011	0.012	0.020
EU086377	0.025	0.019	0.013	0.012	0.006	0.012		0.009	0.011	0.006	0.012	0.019
EU084690	0.038	0.032	0.026	0.025	0.019	0.025	0.013		0.009	0.011	0.015	0.019
EU086389	0.045	0.038	0.032	0.032	0.025	0.032	0.019	0.013		0.013	0.016	0.021
EU086379	0.031	0.025	0.019	0.019	0.013	0.019	0.006	0.019	0.025		0.014	0.017
EU086385	0.025	0.031	0.025	0.013	0.019	0.025	0.025	0.038	0.045	0.031		0.023
KY741876 HS	0.054	0.046	0.039	0.054	0.046	0.054	0.038	0.054	0.063	0.038	0.070	

***same genotype as HQ844231, MG669349, MF398432, JN128898, KC998714

**same genotype as KF176320

*same genotype as EU08468