Knowledge, practices, and epidemiology of bovine fasciolosis in smallholder farming areas of South Africa

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Declaration

I, Sunday Charles Olaogun, declare that the dissertation: "Knowledge, practices, and epidemiology of bovine fasciolosis in smallholder farming areas of South Africa", which I hereby submit for the degree Ph.D in Production Animal Studies, Faculty of Veterinary Science University of Pretoria, is my own work and was undertaken by me from January 2019 to August 2022. All sources used or quoted have been stated, acknowledged, and fully referenced accordingly. This thesis has not been previously submitted by me or any other person for any degree at this University or any other institutions worldwide.

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Dedication

This thesis is dedicated to the Lord Almighty and to my father the late Abisoye Akanji OLAOGUN who passed away during the period of this program. May HIS soul continue resting in peace.

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List of abbreviations

AGDT	Agar Gel Diffusion Test
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
cELISA	Coproantigen ELISA
CU	Copper
ddH2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme Linked Immunosorbent Assay
EPG	Eggs per gram
FAO	Food and Agricultural Organization
FBTs	Food-borne trematodes
FE	Iron
FEC	Faecal eggs count
GDH	Glutamate dehydrogenase
GDP	Gross domestic products
GGT	γ-glutamyl transferase
HB	Haemoglobin
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-4	Interleukin 4
ITS2	Internal transcribed spacer 2
LAMP	Loop Mediated Isothermal Amplification method

ng/µl	Nanogram/microliter
NTDs	Neglected tropical diseases
P	Phosphorus
PCR	Polymerase chain reaction
PCV	Parked cell volume
RBC	Red blood cell
ROS	Reactive oxygen species
SEM	Scanning electron microscope
TGF-β	Transforming growth factor-β
USD	United States dollar
ZAR	South African rand
ZnSo4	Zinc sulphate

Abstract

Knowledge, practices, and epidemiology of bovine fasciolosis in smallholder farming areas of South Africa

Bovine fasciolosis (infection with *Fasciola* species) causes morbidity and mortality in cattle and has zoonotic implications and deleterious economic effects, especially on smallholder cattle farmers. The smallholder farmers' level of knowledge and practices about bovine fasciolosis are not known in communal farming areas of Northwest province. Additionally, the prevalence of bovine fasciolosis, haematochemical changes caused by fasciolosis in cattle, and the species identity and genetic diversity of *Fasciola* species infection in cattle are not known in the communal farming areas of Northwest province, South Africa. The broad objective of this study was to establish the molecular epidemiology of bovine fasciolosis in the communal farming areas of Northwest province, South Africa. The knowledge, attitudes, and practices concerning bovine fasciolosis among smallholder cattle farmers (n = 153) were determined in three villages of the Moretele Local Municipality in Bojanala District, North West Province of South Africa using a structured questionnaire. As a follow-up, a cross-sectional study was conducted to determine the occurrence of bovine fasciolosis in cattle (n = 277) using the sedimentation technique, real-time polymerase chain reaction (qPCR), and faecal antigen enzyme-linked immunosorbent assay (Ag ELISA) in five villages of the Moretele Local Municipality in Bojanala District, North West Province. Furthermore, the effect of *Fasciola* species infection on haematological and biochemical parameters was determined in communally grazed cattle using Auto (ADVIA® 120 Hematology system). Lastly, *Fasciola* species were characterised in infected communally grazed cattle by sequence analysis of the nuclear ribosomal internal transcribed spacer 2 (ITS-2 gene) and the mitochondrial cytochrome oxidase 1 (CO1) regions. No evaluated factors were significantly associated with a positive fasciolosis epidemiological knowledge score. Education level (P = 0.046), cattle breeds being

reared (P = 0.022), and management system (P < 0.001) of the smallholder farmers were associated with a positive practice score concerning bovine fasciolosis prevention. Only 73 (26.40 %) cattle were positive using the qPCR assay while 36 (13.00 %) were positive using the sedimentation technique, though with low feacal egg counts. All cattle samples were negative for bovine fasciolosis using faecal Ag ELISA. Location, breed, sex, age, and faecal consistency score did not affect cattle's qPCR positivity to bovine fasciolosis (p>0.05). The egg per gram load of Fasciola correlated significantly (P < 0.05) with the RBC, Hb, and PCV in *Fasciola*-infected cattle. There was a decrease in glutamate dehydrogenase and an increase in gamma-glutamyl transferase in the infected compared to non-infected cattle. A total of 30 (41.1%) of the 73 PCR positive cattle were infected with *F. hepatica*, 25 (34.2%) with *F. gigantica*, and 18 (24.7%) with both species. *Fasciola hepatica* ITS-2 sequences are grouped into two different clusters, one cluster with reference sequences from China, Libya, and Peru and the other cluster with a sequence from Spain. The *F. gigantica* ITS-2 sequences grouped in one cluster together with sequences from sheep from Libya and cattle from Chad. The *F. gigantica* CO1 sequences grouped with *F. gigantica* sequences from Zimbabwe, while *F. hepatica* CO1 sequences grouped with *F. hepatica* sequences from sheep and cattle from Japan, Tunisia, Austria, and Ecuador. It was concluded that farmers in the study area generally possessed poor epidemiological knowledge and their educational status and system of management influenced their adoption of satisfactory practices of prevention and control of bovine fasciolosis. The prevalence of fasciolosis was relatively high, especially using the qPCR method. Patent *Fasciola* infection was associated with a relative decrease in the values of most of the haematochemical indices but did not significantly alter the erythrogram, leucogram, and serum chemistry of communal cattle. There was co-infection of both *F. hepatica* and *F. gigantica* in cattle in the study areas which may be indicative of future *Fasciola* species hybridization.

Key words: Cattle, Epidemiology, *Fasciola*, Knowledge, Smallholder farmers, South Africa

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Chapter 1: General Introduction

1.1 Background

The livestock sector is important to the effective economic transformation of South Africa and any other country in the world. There is increasing demand for food of animal origin due to the ever-increasing human population (Delgado et al., 1999; Ranganathan et al., 2018). It is a fundamental fact that livestock, in particular, cattle and by-products in the form of meat, milk, offal, cheese, provide the required animal proteins that are necessary to the development of people's dietary status (Sansoucy, 1995; Thorntorn, 2010). Cattle also help in expanding the family's financial base, property protection, soil maintenance and preservation, transportation, traction for land tillage, sustainable agricultural production, family and community businesses, , and social status (Ndoro et al., 2014). Cattle play an essential role in commodities provision for export, such as live animals, meat, milk, and hides, and balance of trade for an improved economy (Tigabu, 2022). Cattle can also be used in ritual and sacrifice offerings, during ceremonies such as marriages, burials, and other festivities (Molefi and Mbajiorgu, 2017).

The beef industry stands as one of the highest contributors to the South African economy. In 2014 alone, the beef industry contributed R22 billion to the agricultural gross domestic product (GDP) of South Africa and employed about 500,000 people (Mdluli, 2019). Commercial producers number about 50,000, emerging farmers about 240,000, and about 3 million communal farmers engaged in beef production and livestock rearing in South Africa (Phaleng et al., 2018). A myriad of challenges faces South African cattle farmers, especially the majority that are emerging and the communal farmers. These challenges include globally increasing production costs, unstable prices of feed grain, persistent drought, diseases of livestock and increasingly difficult food safety policy that pressurize gain and farming products associated with beef globally. With the projected increase in middle-class expenses and population growth

from one billion to two billion people by 2050 in Africa, including their associated red meat demand, every stakeholder must be ready to provide solutions to challenges facing the livestock sector (FAO, 2017). It has been reported that South Africa is second only to Ghana in Africa on meat consumption which is evaluated to be 41 kg per capita per year (Takeuchi et al., 2014). There is an urgent need to provide solutions to many of the challenges to cattle production in which diseases are amongst the most prominent. The neglected helminth tropical diseases such as bovine fasciolosis, feature among the major livestock diseases hampering cattle production in South Africa (Mpisana et al., 2022).

Food-borne trematodes (FBTs) are one of the important classes of neglected tropical diseases (NTDs) globally with more than 40 million people infected and a projection of 750 million (>10% of the world's population) at risk (Zhang et al., 2017). Over 100 species of FBT are zoonotic (Keiser and Utzinger, 2007). The poorest people in rural communities of the endemic countries are at greater risk of trematodosis. Many factors contribute to the high prevalence of these infections, including; low level of education, poor recognition of infected animals due to the subclinical nature of the disease,especially the sub-acute and chronic form of the disease, poverty, concurrent infection, malnutrition, poor food inspection, and poor hygiene (Bottieau, 2021). One of these major trematode infections that interfere with the successful production of ruminants is fasciolosis or liver fluke infection. *Fasciola* infection results in economic losses, which include; liver condemnation at meat inspection, mortality, losses associated with decreased milk and meat production, secondary bacterial infections, reproductive failure, and the cost of frequent anthelmintic treatments (Beesley et al., 2018). The aetiology of fasciolosis are large leaf-shaped flatworms of the class Trematoda from the species *Fasciola gigantica* (*F. gigantica*) and *Fasciola hepatica* (*F. hepatica*). The genetics, behavioral, morphological, and

anatomical structures of both species are closely related. Hybridization of the two species has been previously reported in South Africa by Haridwal et al. (2021).

Fasciola spp. life cycle is indirect and requires the snail intermediate hosts of the family *Lymnaeidae*. Five developmental stages are involved in the liver fluke life cycle namely: miracidia, sporocysts, rediae, cercariae, and metacercariae; the metacercariae is usually the stage infective to the definitive host (Kaplan 2001). Both humans and animals get infected by consuming metacercariae in contaminated water or vegetation. Very rarely can humans get infected by metacercariae in meat. Adult *Fasciola* flukes inhabiting the bile duct release eggs into the gastrointestinal tract, which are defecated into stagnant water (Gonzalez-Miguel et al., 2021). These eggs hatch and develop into the first stage, free swimming miracidia which penetrate the snail host and grow through sporocyst and redia stages into cercariae (Moazeni and Ahmadi, 2016). The second, also free-swimming, cercariae stage is discharged from the snail and gets encysted in freshwater plants or on water surface (Gonzalez-Miguel et al., 2021). Humans or animals get infected with the parasite through the consumption of plants and or water contaminated with metacercariae (Ejeh et al., 2015). The snail intermediate hosts and the presence of *Fasciola* spp. have been reported in various provinces of South Africa (Malatji et al., 2020; Malatji and Mukaratirwa, 2020; De Cock and Wolmarans, 1998). Molecular studies have confirmed the presence of *F. hepatica* in Gauteng (Alves et al., 1988), Mpumalanga and KwaZulu-Natal provinces (Mucheka et al., 2015), and *F. gigantica* in Mpumalanga, KwaZulu-Natal (Mucheka et al., 2015) and Eastern Cape provinces (Malatji and Mukaratiwa, 2020). Both species have been reported to overlap in the provinces of KwaZulu-Natal and Mpumalanga. There is a dearth of published reports on the prevalence of fasciolosis in the North West Province.

Nyindo and Lukambagire (2015) in Tanzania reported the prevalence of fasciolosis to be higher in smallholder cattle herds and attributed this to high illiteracy rates, poor recognition of the disease, limited resources for control, suboptimal nutrition, and poor biosecurity. The high burden of fasciolosis often leads to poor off-take and reduced incomes which are characteristic of smallholder cattle operations (Molefi and Mbajiorgu, 2017). For the effective control of bovine fasciolosis in smallholder cattle herds, it is essential that farmers have proper and adequate knowledge. Moreover, the farmers' attitudes and practices concerning the disease should be given attention, as these will affect the success of implemented control measures.

The importance of clinical signs, grazing history, seasonal occurrence, examination of faeces by laboratory tests, and post-mortem examination in diagnosing bovine fasciolosis have been described (Kebebew et al., 2021). Clinical manifestations of fasciolosis depend on the level of liver damage by migrating immature and established mature flukes varying from acute, characterised by sudden death, to sub-acute, characterised by jaundice, lethargy, mild diarrhea, and anemia, and chronic, characterised by marked anaemia, inappetance, weight loss and submandibular oedema (Lalor et al., 2021; Mitchell, 2020; Williams, 2020). Each phase of the disease may elicit different changes in the haematological and biochemical parameters of cattle and these changes could be used as diagnostic and prognostic indicators in infected cattle (El-Aziem Hashem and Mohammed, 2017). A drastic decrease in the level of packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) with increasing liver fluke load in cattle was previously reported in Nigeria by Egbu et al. (2013). In addition, a significant increase in the level of liver enzymes (gamma glutamyl transferase, alanine aminotransferase and glumate dehydrogenae), urea, and creatinine were also reported in *Fasciola*-infected cattle compared to a non-infected group in Egypt (Nasreldin and Zaki, 2020). Data are, however, scant on the hematological and biochemical changes associated with bovine fasciolosis in

cattle, reared in smallholder farming areas in the Northwest province, South Africa as related to breed, sex, age, and physiological status.

Fasciolosis diagnosis can be tentatively established based on background epidemiological information/knowledge of the disease in a particular area, observed signs, grazing history information, and available data on seasonal variation of occurrence (Mitchell, 2002). Confirmatory diagnosis is based on the demonstration of *Fasciola* eggs in faeces through coprological examination utilising the sedimentation technique or by demonstration of immature and mature flukes in the liver at post-mortem examination (Happich, 1969; Urquhart et al., 1996). Though these two tests are effective, they detect liver fluke after it has caused significant pathology and thus, they may not be useful for early on-farm detection of the disease. The sedimentation method has limitations as an antemortem diagnostic test as it is laborious, has low sensitivity especially when faecal egg counts (FEC) are low, cannot differentiate between the different *Fasciola* spp., and cannot detect pre-patent infections (Brockwell et al., 2013). Serological techniques based on antibody detection have been adopted to detect animals' exposure to liver fluke in many countries and possess the benefit of relatively high sensitivity (97–100%) and specificity (96–100%) and the ability for quick screening of many animals (Martínez-Sernández et al., 2018). The major disadvantage of antibody-based serological tests is that they cannot distinguish between current infection and previous exposure as antibodies persist even when the infection has been cleared naturally or by anthelmintic drug administration (Simões et al., 2017).

The faecal (or copro-) antigen enzyme-linked immunosorbent assay (coproAg ELISA) has also been developed to detect *Fasciola* spp. secretory-excretory antigens in faeces even during the pre-patent period and for monitoring the efficacy of treatment, post-anthelmintic administration (Martinez-Sernandez et al., 2018). The coproAg ELISA however, cannot

differentiate between the two *Fasciola* spp. and has low sensitivity when FEC is low (Brockwell et al., 2013; Martinez-Sernandez et al., 2016). Recently Calvani et al. (2017) developed a quantitative real-time polymerase chain reaction (qPCR) that is highly sensitive and specific, detecting *Fasciola* spp. deoxyribonucleic acid (DNA) in faecal samples even with very low egg counts and as early as two weeks post-infection (Martínez-Pérez et al. 2012; Robles-Perez et al., 2013). This diagnostic test has the potential to be used in epidemiological studies of fasciolosis in cattle. The tests capable of antemortem detection of fasciolosis have however not been compared to select the most appropriate test, in terms of sensitivity, specificity, and practicability, for prevalence studies in cattle, especially under natural field conditions in South Africa.

To characterise *Fasciola* parasites to the species level, morphometric techniques have been applied to adult flukes recovered on postmortem liver examination including the width-to-body length ratio (Itagaki et al., 2009). The adult *Fasciola gigantica* worm is longer than it is wide whereas *F. hepatica* has a higher width-to-body length ratio. Morphometric techniques can be inaccurate due to the variations in fluke size, as influenced by the age of flukes, involved host species, and the fixation technique adopted (Ichikawa and Itagaki, 2012). Furthermore, these techniques cannot be accurately applied in the ante mortem characterisation of other life stages of *Fasciola* such as eggs in faecal samples. Additionally, the presence of intermediate forms or hybrid *Fasciola* spp. has led to confusion with phenotypic characterisation using morphometric techniques (Le et al., 2008; Haridwal et al., 2021). Modern morphometrics using geometric morphometrics and artificial neural networks for enhanced morphological identification have been developed (Sumruayphol et al., 2020). These however have limited application due to their demands on skills and time, and an inability to differentiate hybrid forms. To overcome the limitations of morphometric characterisation, PCR-based methods,

utilizing nuclear or mitochondrial DNA targets, have been used in the genetic characterization of *Fasciola* spp. (Alasaad et al., 2011; Le et al., 2012; Mas-Coma et al., 2009).

Rojas et al. (2014) reviewed the molecular DNA-based approaches used to characterize different species of *Fasciola*. Molecular methods have ranged from random amplification of polymorphic DNA and microsatellite analysis ((Hurtrez-Bousses et al., 2004) to PCR-restiction fragment length polymorphism (RFLP) (Ichikawa and Itagaki, 2010), and phylogenetic analyses of mitochondrial gene regions and internal transcribed spacers (ITS) (Mas-Coma et al., 2009). Some authors have used quantitative real time PCR (qPCR) and species-specific probes to identify *Fasciola* spp. (Alasaad et al., 2011; Calvani et al., 2017). These methods have the added advantage of being applicable to any life stage of the flukes and for ante mortem diagnosis. The species of *Fasciola* infecting cattle in the communal farming areas of North West province have, however not been genetically characterized and no previous documentation has been deposited in the Genbank.

Effective sustainable control measures for fasciolosis must be based on a proper understanding of the relationship between the parasite, hosts, and the environment (Integrated parasite management). Available literature shows that there are no chemicals currently approved for the control of the snails which are the intermediate host of *Fasciola* spp. (Roberts, 1996). Hence fasciolosis control efforts must be channeled at reducing exposure of the animals to infection and prophylactic treatment of exposed animals. Generally, herd-based control of fasciolosis involves four major aspects: enhanced drainage, fencing off muddy areas during risky seasons, periodical screening of the herd for fluke infestation using faecal egg counts and antibody levels detected in the bulk milk tank, and routine treatment in endemic area and control of stocking density (Nielsen et al., 2021). However, the interpretation of derived results must be evaluated carefully. Negative egg counts may not denote freedom from infection as it may be

falsely negative. Also, appropriate biosecurity measures which may involve avoiding the introduction of infected animals into the herd and/or implementing strict isolation/quarantine of introductions until four weeks have elapsed after treatment with a flukicide effective against immature stages can be adopted as control measures.

The treatment of fasciolosis in animals is the most reliable control measure. McNair (2015) reported that the rate and the spread of fasciolosis had increased and there has been evidence of resistance to available anthelminthic or drug efficacies diminishing.

Effective control and management of fasciolosis generally depend on general understanding of the epidemiology of the disease by the farmers.

1.2 Problem statement

The knowledge, attitudes, and practices (KAP) of smallholder farmers on bovine fasciolosis have been used to assess their willingness to adopt prevention and control measures (Tiongco et al. 2012). Assessment of farmers' KAP on bovine fasciolosis is essential for the development of appropriate policies and strategies to prevent and control the disease (Aregahagn and Melkamu, 2018). Studies on communal farmers' knowledge, attitudes, and practices about bovine fasciolosis in the North West Province of South Africa, however, are scarce and require investigation.

There is a paucity of information on the current prevalence, and molecular epidemiology of bovine fasciolosis among cattle reared in smallholder farming areas of the North West province of South Africa. The overall occurrence of bovine fasciolosis and the influence of age, sex, breed, and season as associated risk factors for fasciolosis in cattle has not been elucidated in

the North West Province of South Africa. Ante mortem detection of *Fasciola* spp. in cattle has been achieved using sedimentation and faecal egg counts (FEC), faecal Ag ELISA, and quantitative PCR (Rojas et al., 2014). There is a present need to compare diagnostic methods for the selection of the most sensitive and specific on-farm method to detect the prevalence of bovine fasciolosis under natural conditions. Field-based prevalence studies will provide essential ante-mortem data for the development of fasciolosis control strategies that will mitigate economic losses.

Fasciola parasites exert their main pathological effect by mechanical and toxic effects on the vascular and biliary systems of the liver (Lalor et al., 2021). The changes in the haematological parameters associated with *Fasciola* infection in cattle include reduced haemoglobin (Hb), red blood cell count (RBC), packed cell volume (PCV), lymphocytes, and monocytes with increased total white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils, and eosinophils (Brahmbhatt et al., 2021). *Fasciola* infection has been shown to significantly lower levels of total protein (TP), albumin (Alb), and glucose (Glu) while increasing the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) in infected animals (Kozat and Denizhan, 2010). There is however, a lack of information on the haematology and biochemistry indices of *Fasciola*-infected in comparison with non-infected cattle in the North West Province of South Africa.

To accurately identify *Fasciola* spp., especially when dealing with unknown forms with distinct phenotypes and genotypes, it is important to use molecular analyses as identification tools (Hayashi et al., 2015). Sequences of the internal transcriber spacer 2 (ITS-2) and cytochrome C oxidase subunit 1 (CO1) gene have been employed in the phylogenetic

classification of *Fasciola* spp. in previous studies (Omar et al., 2013; Mucheka et al., 2015; Zishiri et al., 2019). The genetic diversity of the two *Fasciola* spp. in the North West province of South Africa is still poorly understood, and there is currently no knowledge of the existence and sequence variation of the two *Fasciola* spp. in the North West Province. Studies on animal fascioliosis are not plentiful in South Africa and available data were mostly from abattoir-based reports with very few on-farm studies. All these and other factors contribute to the need to conduct extensive studies on farmers' awareness, prevalence, associated risk factors, diagnosis, and molecular epidemiology of fasciolosis in South African cattle owned by communal and emerging farmers in the North West province.

1.3 Justification

The importance of bovine fasciolosis as relating to cattle productivity and public health cannot be overstated. Fasciolosis continues to be associated with low-resource farming systems and this poses a challenge, especially in cattle reared under smallholder farming operations which carry almost half of the cattle population of South Africa. Understanding the knowledge, practices, and attitudes of communal and emerging farmers is a key step before investigating the disease burden in cattle and developing intervention strategies to prevent and control bovine fasciolosis at the herd level.

Despite the presence of large numbers of cattle, the abundance of the snail intermediate host, and appropriate weather and climatic conditions that favor the excessive proliferation of *Fasciola* spp., studies on bovine fasciolosis are limited in South Africa. Current data on the prevalence of bovine fasciolosis are derived from abattoir-based studies. There is a dearth of on-farm studies focusing on the prevalence of fasciolosis and associated risk factors, especially in communal and emerging farming areas of South Africa. Determining the prevalence, risk

factors, and diversity of *Fasciola* species utilizing highly sensitive and specific methods such as PCR in cattle could provide baseline data for the development of control measures based on sound scientific evidence in communal and developing farming areas. There is a need to understand the presence of clinical and subclinical fasciolosis and associated biochemical and hematological changes in cattle, to make inferences on the host-parasite relationships that lead to resilience to the disease in different bovine hosts.

The information and data to be generated by the present study will assist to raise awareness of bovine fasciolosis among the farmers, veterinarians, livestock health providers, and the general public of South Africa. Findings from this study will also serve as a basis for future research on bovine fasciolosis, prevention of anthelminthic resistance, and possibly the development of vaccine candidates for fasciolosis in cattle. Data generated from this study may be used by the stakeholders in the animal health sphere and government to formulate a national policy for fasciolosis control in cattle in South Africa.

1.4 Aim of the study

The broad aim of this study was to assess the knowledge, attitude and practices of smallholder farmers on bovine fasciolosis as well as to determine the disease prevalence, associated risk factors, species identity, and genetic variation of *Fasciola* species in cattle reared under smallholder farmer management systems.

1.5 Study objectives

1. Assess the level of knowledge, attitudes, and practices on bovine fascioliosis of smallholder cattle farmers in the North West Province of South Africa, utilizing questionnaires.
2. Establish the prevalence and associated risk factors of fascioliosis in cattle reared by smallholder farmers in the North West Province using different diagnostic methods.

3. Compare the hematological and biochemical parameters of *Fasciola*-infected and non-infected cattle reared by smallholder farmers in the North West Province.

4. Determine the species identity and likely genetic variation of different *Fasciola* species using molecular typing techniques in cattle reared by smallholder farmers in the North West Province.

1.6 Hypotheses

The specific hypotheses that were tested include:

1. Communal and emerging cattle farmers in the North West Province of South Africa possess poor knowledge, attitudes, and practices on the epidemiology of bovine fascioliosis.

2. The prevalence and associated risk factors of bovine fascioliosis are are low in cattle in cattle reared by communal and emerging farmers.

3. There is no significant difference in the hematological and biochemical indices of *Fasciola*-infected and non-infected cattle reared under the communal and emerging farmer management systems.

4. Smallholder cattle the North West Province of South Africa are infected by only one species of *Fasciola* with no genetic variation.

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Chapter 2: Literature Review

2.1 Introduction

The importance of the cattle farming sector to the South African economy cannot be over-emphasized. A substantial percentage of cattle farmers in the country are engaged in smallholder cattle farming (Oduniyi et al., 2021). The smallholder cattle farmers comprised of communal and emerging farmers, encounter a myriad of challenges in which animal diseases are an important problem. Bovine fasciolosis features among the major livestock diseases hampering cattle production in South Africa (Mpisana et al., 2022). Fasciolosis is also a re-emerging and well-known zoonosis impacting a huge proportion of people worldwide (Mas-coma et al., 2005). The disease causes direct and indirect economic losses to the cattle industry in South Africa (Jaja et al., 2017). Despite the importance of communal cattle production in the North West Province of South Africa, there is limited information on farmers' awareness of bovine fasciolosis and the epidemiology of the disease in the province. It is, therefore, imperative to explore the epidemiology of bovine fasciolosis, considering the agricultural potential and involvement of the smallholder sector in animal production, especially in the communal farming areas of the North West Province. The present review focuses on general information on the epidemiology of fasciolosis and the current state of bovine fasciolosis in the North West Province of South Africa.

2.2 Cattle production in South Africa

The animal rearing sector in South Africa is an important contributor to the nation's agricultural trade, contributing over 48 % of the total agricultural output (Mapiye et al., 2018). Between the period of 1995/2000 to 2006/2010, approximately 185 % more cattle contributed to South Africa's GDP than before, and livestock products compared to field crops and horticulture improved their contribution to the agricultural GDP from 42 % to 47 %.

Around 70 % of available agricultural land in South Africa is only suitable for raising livestock and these land used is divided intosubsistence smallholder farmers (referred to as communal farmers), market-focused subsistence smallholder farmers (previously known as emerging farmers), and commercial farmers (Hlophe-Ginindza and Mpandeli, 2021). Communal farmers are subsistence-oriented and do not possess exclusive land tenure, but share and collectively manage their natural resources such as grazing land (Palmer, 2006; van Averbeke and Mohamed, 2006). The smallholder farmers that are focused on the market are in the transitional stage from subsistence to commercial farming, having benefitted from the government's land reform program and owning private pieces of land (Ainslie et al., 2002). Commercial farmers farm on large privately owned pieces of land, utilizing technology, have high turnover and profits, and are purely market-oriented (Greenberg et al., 2022). The latter farmers are not the focus of this study.

Cattle farming is a relatively large agricultural subsector in South Africa with a share of 26.2 % (Scholtz et al., 2008). Beef cattle constitute the bulk (12.5 million cattle) of the almost 14 million cattle in South Africa while dairy cattle constitute 1.6 million cattle (604,781 cows in milk). In the beef cattle production system, approximately 53 % of cattle are kept in the commercial sector, and the remaining 47 % are in the smallholder sector (Nyamushamba et al., 2017).

2.3 Communal cattle production in South Africa

Communal farming in South Africa contributes to livelihoods and economic relief for the rural populace who are poverty-stricken. This is entirely dependent on family income and was developed so that community farming families might become economically empowered that, among other things, could use family labor without paying any wage. They rear only breeds that require a low cost of sustenance and breeds that are hardy and can survive under

unfavorable conditions (Mmbengwa *et al.,* 2015). It appears to be generally agreed that communal settings in South Africa are not uniform in nature and differ at a variety of scales, including local, regional, and agroecological zones. Forty-one percent of beef cattle, 12 % of sheep, and 67 % of goats are kept at a small communal scale. South Africa's cattle production is continuously evolving; there is a need to manage diseases that can have the potential to affect productivity, and public health and lead to food wastage (Mmbengwa *et al.,* 2015).

2.4 Cattle breeds and production system in North West province of South Africa

The cattle population in the North West province of South Africa is about 12.9% of the national herd (Meissner *et al.,* 2013). Subsistence and market-oriented subsistence farmers collectively own about 40% of the beef cattle population in the North West Province, and the remaining proportion is owned by farmers in commercial operations (Mapiye et al., 2018). There are about 495 abattoirs and 70 feedlots, with the largest of the feedlot hosting 120,000 cattle and processing 1600 slaughtered animals daily (Motiang and Webb, 2016).

. The commonest cattle breed in North West Province includes Nguni, Afrikaner, and Bonsmara breeds. These indigenous breeds adapted to the prolonged drought condition in the province and previous traits studies revealed above-average in their traits performance and thrive well with minimal management inputs. These breeds performed an important role in beef cattle production, especially with the extensive (bush grazing) farming commonly practised in the North West province (Maime, 2015). According to Schwalbach et al. (2001), breeding females and replacement heifers dominate herds owned by communal farmers in the North West Province while young bulls and oxen make up the balance. Despite the importance of communal cattle production, its current contribution to the national economy is poor due to low off-take caused by amongst others, diseases, and parasites (Mthi et al., 2020). Bovine

fasciolosis features among the major livestock diseases hampering cattle production in South Africa (Mpisana et al., 2022).

2.5 Fasciolosis

According to Jaja et al. (2017) in their work on estimated losses linked to *Fasciola* infection in slaughtered cattle in three abattoirs in Eastern Cape province in South Africa, fasciolosis is one of the prevalent, serious illnesses harming the nation's cattle productivity which often results in a high rate of condemnation of meat and offal as unwholesome for consumption by humans.

2.5.1 Aetiology, classification, and morphology

The parasitic zoonotic disease fasciolosis is caused by trematodes of the genus *Fasciola*, which includes the two species of liver flukes, *Fasciola hepatica* and *Fasciola gigantica* as well as a hybrid of these two species, which are the important etiology of the disease in Africa (Mas-Com et al., 2005).

Morphologically, *F. gigantica* has a flat-shaped body and is more elongated than *F. hepatica*. The adult worm measures 75 mm by 15 mm, and the anterior end is large and cone-shaped with a slopping shoulder and rounded posteriorly (Figure 1). The trematode possesses both oral and ventral sucker for feeding and adhering to the inside of its host, and possesses three different types of surface papillae which are used as sensory receptors. *Fasciola hepatica* measures 30 mm by 15 mm and has a broad shoulder, which demarks the conical anterior part of the entire body (Figure 1). The adult *F. hepatica* is grey-brownish in color, leaf-shaped, and has a flattened body appearance. The immature flukes are about 1-2 mm at the stage of entering the liver (Phalee *et al.*, 2015).

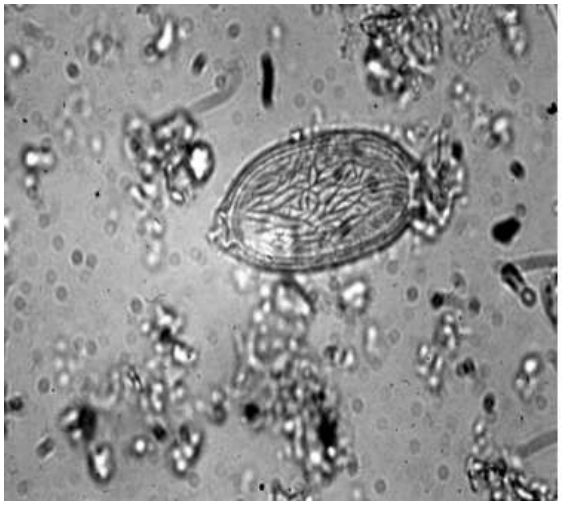
The length of *F. gigantica* eggs is up to 0.2 mm (Longstaffe, 1984). Eggs of *F. gigantica* are thin shelled, large, yellowish, and operculated in appearance. Both the flat operculum and the

umbilicus-like invagination at the shell's terminal end were clearly visible. The outer surface of the eggshell appeared to be smooth and devoid of any micro spines with densely noticeable umbilicus-like invagination on the shell on the other side of the operculated end when viewed under SEM. Sometimes, this umbilicus contains little pieces of debris that appear to have distinct shapes in SEM images. Some eggs may appear to have a knob when viewed under a light microscope due to this debris.

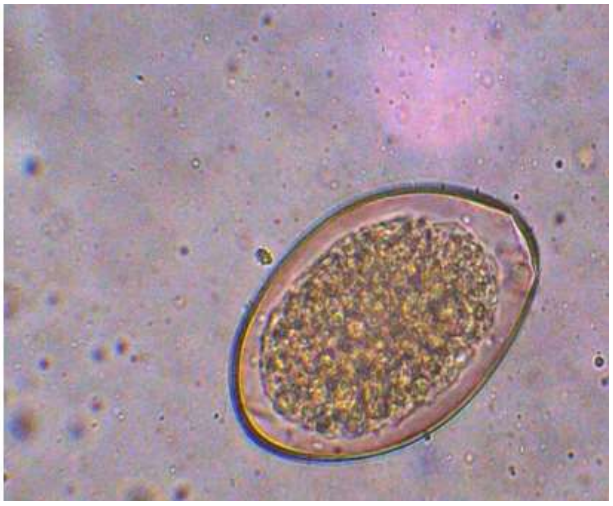
Fasciola gigantica adult

Fasciola hepatica adult

Figure 2.1. Morphology of adult *Fasciola* species (Narva *et al*., 2011)



F. gigantica



F. hepatica

Figure 2.2. Morphology of the eggs of *Fasciola* species (x 40magnification)

(indicate the magnification if taken by you)

2.5.2 Life cycle

Fasciola has a three-part life cycle that includes the final host where adult flukes develop, the intermediate host where larval stages develop, and a carrier that includes freshwater plants. Five stages are recognized during the life cycle, namely eggs, miracidia, cercariae, metacercariae, and adult flukes. The climate (sufficient moisture and temperature) as well as the presence of appropriate intermediate snail hosts and definitive mammalian hosts are all necessary for the life cycle to be completed (Fairweather, 2011).

When mammals consume vegetables (in humans) or pastures (in animals) contaminated with metacercariae, they get infected (Figure 3). The consumed metacercariae encysts in the duodenum before entering the peritoneal cavity through the intestinal mucosa. The cercariae reach the liver and grow into immature flukes, which wander in the the liver' parenchyma feeding on blood and damaging tissue before moving into the biliary ducts, where they mature into adult flukes. In cattle, the development of adult flukes from ingestion of metacercariae takes about 3 to 4 months (Mas-Coma *et al.,* 2014). The immature eggs that are produced by the adult flukes move from the bile ducts into the duodenum and then ejected into the environment via the hosts' faeces. Eggs only begin to develop and embryonate after being released from excreta and needs an aquatic environment for this to occur

The spread of the released eggs is facilitated by break up offaeces by rainfall, or spread of faecal materials by animals or inanimate objects. Under optimal temperature, humidity, and oxygen conditions, for *F. hepatica*, the embryonated eggs hatch into miracidia over a period of

9 to 12 days at a temperature of 22 to 26 °C, while *F. gigantica* eggs take a little longer (about 17 days). There is little or no development at temperatures less than 10˚C. (Lalor *et al*., 2021). Within a few hours, the miracidia penetrate a suitable snail intermediate host (Figure 3) by releasing proteolytic enzymes, and then migrate to the hepato-pancreas as sporocysts which then develop into rediae and then cercariae. The freshwater cercariae, which have a tail, are released from the snail and encyst as metacercariae (which lack a tail)in an aquatic environment. This cystic stage in the last mammalian host is an infectious stage (Affroze *et al*., 2013). Full maturation of *F. hepatica* snail larvae is attained in 30 days at 25°C and 80 days at 15°C temperature.

Aquatic plants such as watercress and water mint are normally used by the cercariae to encyst forming metacercariae, however they can encyst on other aquatic vegetation . Ingesting free metacercariae that are floating in water has the potential to be a method of transmission (Slifko *et al*., 2000). In the temperate region, the peak development of metacercariae on pasture is usually in late summer and autumn in a particular year, it is usually a function of the prevailing climatic condition. (Moazeni and Ahmadi,2016).

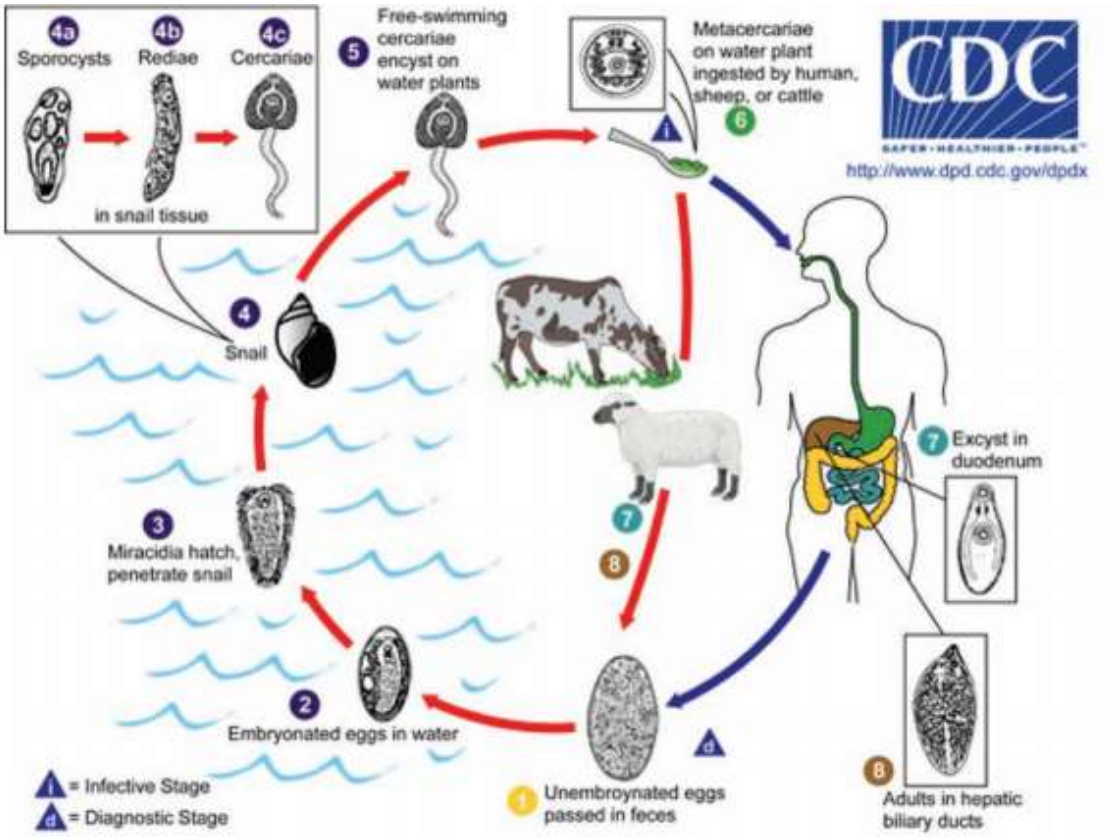


Figure 2.3. The environmental life cycle of Fasciola spp, along with a transitional snail and permanent mammalian hosts (Narva *et al*., 2011)

2.5.3 Snail intermediate hosts

Asexual reproduction occurs in the Lymnaeidae gastropod freshwater snails, which serve as intermediate hosts for *Fasciola*.. These snails are members of the phylum Mollusca and class Gastropoda, which also include freshwater, marine, and terrestrial snails and slugs; subclass Euthyneura and Pulmonata, superfamily Lymnaeoidea and family Lymnaeidae, genus *Lymnaea* (usually *Galba truncatula*). *Galba*, *Radix*, and *Pseudosuccinea* (previously known as *Lymnaea*) snails are thought to play a major role in the transmission of *Fasciola* species (Thanh, 2012). There are over 20 species of Lymnaeidae that have been identified as potential intermediate hosts for *Fasciola*, with varying transmission rates and geographic distribution,

environmental elements (such as temperature and rainfall), and climatic factors (such as habitat and substratum type) (Torgerson and Claxton, 1999).

Galba, *Radix*, and *Pseudosuccinea* (previously known as *Lymnaea*) snails are thought to play a major role in the transmission of *Fasciola* species (Thanh, 2012). The most effective intermediate hosts for *F. hepatica* are *Galba truncatula* and *Pseudosuccinea columella*, while the primary hosts for *F. gigantica* are *Radix natalensis* and *Radix auricularia* (Brown, 1994). According to De Kock *et al*. (2003), *Galba truncatula* is widespread in temperate climates of North America, Australia, and particularly Europe, but *Pseudosuccinea columella* is widespread and is native to the eastern United States and Central America (Cruiz-Reyes and Malek, 1987), and was successfully introduced to Africa, Europe, Oceania and South America (Mas-Coma *et al.*, 2005). According to descriptions of the distribution and habitats of the snail that serves as the intermediate host for the liver fluke in South Africa, the liver fluke's distribution and habitats are related to the snail's amphibious lifestyle in general, its adaptation to cooler habitats, and its highly resilient capacity to withstand drought and other extreme environmental conditions frequently found in unstable water bodies (Mahulu *et al.*, 2019). According to De Kock and Wolmarans (2008), *G. truncatula* transmits *F. hepatica* and is primarily found in cooler regions of South Africa, whereas *R. natalensis* is the intermediate host of *F. gigantica* (Moema *et al.*, 2008) and *P. columella*, the intermediate host of both *F. hepatica* and *F. gigantica* is widely distributed in the country (De kock and Wolmarans 2008; Perissinotto *et al*., 2014). *Radix auricularia* was recently discovered for the first time in the South African province of KwaZulu-Natal (Malatji *et al.*, 2019). The frequency of fasciolosis in livestock has increased as a result of the introduction of *P. columella* to South Africa (Brown, 1994). Experimental research in Cuba revealed that while some *P. columella* strains were resistant to *F. hepatica*, others were vulnerable (Gutiérrez *et al*., 2003; Gutiérrez *et al*., 2005).

In Egypt, *P. columella* was successfully infected with *F. hepatica* in an experimental study (Dar et al., 2015). In France, an experimental comparison for *F. hepatica* metacercarial production between *G. truncatula* and *P. columella* showed a significantly higher production with *P. columella* than *G. truncatula* (Vignoles *et al*., 2015) which could imply greater effectiveness of *P. columella* as an intermediate host for *F. hepatica* than *G. truncatula.* Species of snails that have been previously reported in South Africa are *G. truncatula* (*G. umlaasianus*), *R. natalensis*, and the invasive *P. columella* (De Kock and Wolmarans, 2008).

Galba truncatula (Ohari *et al*., 2017)

Galba truncatula *Galba mweruensis*

Figure 2.4. Morphology of important intermediate snail hosts for *Fasciola* in South Africa (Ohari *et al*., 2017)The distribution of both species of *Fasciola* follows that of the snail intermediate hosts found in both the warm and cold regions (Prasad *et al*., 2008). Because of geographic overlap or livestock mobility, adults of different species may coexist in the same animal host (Mas-Coma *et al*., 2009). There have been previous reports of hybrid species due to the interbreeding of the two *Fasciola* species (Agatsuma *et al*., 2000; Ashrafi *et al*., 2006). Some African and Asian nations have distribution overlaps that lead to the hybridization of *Fasciola* species and the presence of intermediate forms of the parasite in nations like Japan, Iran, and Egypt (Periago *et al*., 2006). Due to the introduction of its intermediate host, *G. truncatula*, from European nations to other continents, *F. hepatica* has expanded to new locations (Mas-Coma *et al*., 2005). In Egypt, where both species are found, morphologically intermediate variants of the two species have been reported (Periago *et al*., 2008). It has been challenging to distinguish the different hybrid species in nations like Japan, Taiwan, and Korea (Marcilla *et al*., 2002). In several Asian nations, morphological characteristics that resemble both species and transitional forms have been recorded (Marcilla *et al*., 2002). Due to morphological heterogeneity caused by this distribution overlap, it is difficult to differentiate the species of *Fasciola* in these nations, which has caused disagreement about their taxonomic categorization (Periago et al., 2008). A number of variables, including climate change, unrestricted transboundary animal movement, and bad land management procedures, have been connected to this distributional drift (Kenyon *et al*., 2009).

2.5.4 Epidemiology and risk factors

2.5.4.1 Host range of Fasciola species

Fasciolosis mainly affects domestic and wild ruminants (sheep, cattle, goats, buffalo) and also humans (Afshan *et al*., 2014). *Fasciola gigantica* is more adapted and infective to cattle than other ruminants, while laboratory animals are not readily infected (Mochankana and Robertson, 2016). On the other hand, *F. hepatica* causes disease in both cattle and sheep with devastating economic consequences. In humans, the disease is recognized in most developing countries as an emerging and neglected disease (Nguyen *et al*., 2011).

2.5.4.2 Global distribution and prevalence of Fasciola species

Fasciolosis is regarded as a neglected re-emerging zoonosis in several parts of the world, including South Africa (Caravedo and Cabada, 2020). *Fasciola gigantica* is commonly found in Africa and Asia, especially in tropical and warmer areas, while *F. hepatica* is mainly found in temperate countries, where it is the most common cause of liver fluke disease.

2.5.4.3 Climate, climate change, environment and seasonality

Previous finding reveals the effects of recent changes in environmental and climatic conditions on a wide range of infectious organisms, vectors, and reservoir hosts viz-a viz it effects on the well-being and health of humans and animals.Many parasitic infections rely on circadian rhythms to guarantee that transmission phases are available at the same time as the host is exposed to the intermediate host or vector, especially those whose life cycles involve a vector or intermediate host. Climate change may cause asynchrony by interfering with the availability of the snail's intermediate hosts in the event of fasciolosis at the time necessary to finish the organism's life cycle. Every scale of biological, social, ecological, and geographic organization will likely be affected by climate change. Because the life history characteristics of the *Fasciola* organisms involved in fasciolosis' life cycle are linked to a particular environmental situation,

local-scale factors are crucial for understanding how climate change will affect fasciolosis (Booth, 2018).

Differences in seasonal prevalence of fasciolosis in South Africa have been reported where the seasonal variation in prevalence was attributed to likely factors such as ambient temperature, solar radiation, and other climatic condition alteration that favors the multiplication of intermediate host causing high infection in cattle (Jaja *et al*., 2017). According to reports from Germany, the percentage of grassy regions and the percentage of water bodies were both found to be significant predictors of *F. hepatica* infection (Mas-Coma *et al*., 1999). In contrast, rainfall and temperature are the major predictors of *F. hepatica* in England, Wales, and Ireland, along with soil structure and minerals (Selemetas *et al*., 2014). There have also been some identified seasonal and demographic risk factors for fascioliasis in domestic ruminants. Due to the free-living stages of *F. gigantica* and the intermediate snail host *Lymnaea auricularia* var. rufescens, fascioliasis epidemiology has a spatial component (Rahman *et al*., 2017) and the influence of climatic and environmental conditions (Molento *et al*., 2018).

2.5.4.4 Vegetation and water plant species

The roles played by vegetation and water plant species in the spread and transmission of fasciolosis, especially human fasciolosis have long been established in most developed countries of the world. Consumption of freshwater consumable vegetables attached to infective *Fasciola* metacercariae has been linked to human infection (Mas-Coma *et al*., 2018). It was recognized that certain aquatic plants that were consumed by humans and used in salads and dish accompaniments, such as watercress, dandelion, and occasionally other plants that carried metacercariae, might infect people (Mas-Coma *et al*., 2018). The ecology of the lymnaeid vectors in a specific area of endemicity determines the plants that the fasciolid cercariae choose to attach to and encyst to become metacercariae, according to both field investigations and

laboratory experimental work. The vegetables usually selected by the lymnaeids cercariae are those growing in stagnant or slowly running waters. Other factors responsible for the presence or absence of the lymnaeids vectors are the existence of salt in the water and the shadow area within the water body (Mas-Coma *et al*., 1999).

2.5.4.5 Soil and topography

Roles of soil constituent, nature, and topography in the endemicity of fasciolosis have been described by some authors especially the analysis of soil minerals as a strong or weak predictor of Fasciola infection. Environment and climate variability were analysed in Swedish beef cattle to identify *F. hepatica* high-risk areas and characterize potential risk factors for *F. hepatica* exposure in beef cattle herds in Sweden (Novobilský *et al*., 2015). Iron concentration was confirmed as a positive predictor and Copper and Phosphorus concentrations were both negative predictors for *F. hepatica* exposure in minerals model analysis of soil minerals in Sweden beef cattle herds (Novobilsky *et al*., 2015).

2.5.4.6 Community factors

Community variables have a crucial role in the transmission of fasciolosis in the field since the disease is transmitted through freshwater, which are the habitat of the lymnaeid vectors. Prevalence and endemicity of *Fasciola* infection in any area is a function of the distance of the farm or/and village from the closest river, dam, or stream inhabited by lymnaeid snail vectors. Due to the ingestion of vegetables containing metacercariae, human infection in metropolitan areas only occasionally occurs, mostly at home and infrequently at restaurants and hotels. Urban dwellers can also acquire the infection from an uncontrolled market that receives vegetables directly from the field. The possibility of infection is also common during field trips (Mas-Coma *et al*., 2018).

2.5.4.7 Mammalian host factors

Age

Dereje *et al*. (2018) described risk factors associated with bovine fasciolosis as breeds, sex, age, season, herd size, and herd composition in Ethiopia. Chakraborty and Prodhan (2015) described sex as a risk factor for bovine fasciolosis in Bangladesh.

An earlier investigation in South Africa found that older animals had much lower infection rates than younger ones (Jaja *et al*., 2017), young cattle not previously exposed to grazing metacercariae-infected pastures, may have a higher infectivity rate compared to older animals that have been previously exposed and developed some level of immunity. Furthermore, the high immunogenicity of the parasite, which supports the development of acquired immunity, may be the cause of the older cattle's poor susceptibility or may be associated with the previous infection in older animals (Khan *et al*., 2010). However, some studies reported a significantly higher prevalence of Fasciola in older animals as compared to young animals (Maqbool *et al*., 2002; Pfukenyi *et al*., 2006). Therefore, it will be difficult to state in clear terms if infectivity or susceptibility is higher or lower at any particular age. Only to state that it has been established that previous exposure or infection leads to the development of short-term acquired immunity protecting older animals.

Breed

The occurrence of fasciolosis has been reported to be more in the local breeds of cattle when compared with the exotic breeds. Several logical reasons have been stated to justify this, firstly local breeds are owned mostly by poor-resourced rural/communal smallholder cattle farmers who most often will not have access to veterinary care and improved farming technique compared with a well-organized commercial farming system with modern veterinary care and farming technique with reduced incidences of fasciolosis (Musemwa *et al*., 2008).

Furthermore, local breeds are often on extensive grazing system, where they graze in open fields and drink from either dams, ponds, streams or rivers which are often contaminated or infected with *Fasciola* metacercariae. Lack of proper disease control infrastructure and knowledge on the part of the owners of these local breeds make them more prone to Fasciolosis and even other diseases (Soji *et al*., 2015).

Sex

Female animals were reported to be more infected than their male counterparts in general (Khan *et al*., 2010). One would believe generally that female animals should have less exposure to contaminated pasture due to more care always provided and a higher trend of stall feeding compared to males, but stress and decreased immune status associated with pregnancy and periparturient period have been linked to a higher infection rate of parasitism generally in female animals than males (Urquhart *et al*., 1996). In another school of thought, Opio et al. (2021) explained that the relative longevity of females compared to males is due to the advantages of maximization of their reproductive capacity and their milk production potential. This would increase the risk of contracting the disease in females more than in male animals. However, a higher prevalence was reported in male ruminants than in females, where most of the males were kept under grazing practice and females were not grazed during pregnancy (Raza *et al*., 2007).

2.5.5 Diversity of *Fasciola* species

Several authors have used molecular markers to describe the variety of *Fasciola* species, phylogenetic clustering, and sequence variation of their genes. There have been previous reports of simultaneous infection with both species in cattle in northern Iran and overlap in the spread of both species in Asia (Karimi, 2008). Using phenotype criteria in differentiating *Fasciola* species seems very unrealistic. Therefore, molecular genetic techniques based on

nuclear and mitochondrial DNA have been used to identify, characterize, and in phylogenetic analysis of the parasite (Gasser, 2006). A long-running debate over the taxonomic identity of these species, which are found in some countries, particularly Iran, Egypt, Japan, Taiwan, the Philippines, and Korea, and in which a wide variety of morphological types is detected, has even been sparked by the overlapping distribution of both *F. hepatica* and *F. gigantica* (Mas-Coma *et al.*, 2005). The rDNA sequence gives genetic markers for the correct identification and differentiation of *Fasciola* species, according to earlier research. The sequence of well-defined 18S rDNA of *Fasciola* species has been used to identify and characterized *Fasciola* species in Iran (Karimi, 2008). Analysis of the ITS2 and d2 regions revealed polymorphism, meaning that two of the five *Fasciola* had an *F. gigantica*-type in a sequence, an *F. hepatica*-type sequence, and two of them had sequences of both kinds, indicating that the loci harbour several alleles. Using a phylogenetic tree, one could detect a close relationship between isolates in a particular country with those of other countries. The phylogenetic tree of ITS 1 described by Aryaeipour *et al.* (2014) indicated that flukes were dispersed as pure *F. hepatica* and *F. gigantica* clades, suggesting that two *Fasciola* genotypes can infect animals and perhaps humans in north-western Iran.

2.5.6 Pathogenesis, clinical signs and pathological findings

2.5.6.1 Pathogenesis

The pathogenesis of the disease is a function of the number of metacercariae ingested over time. Within 7 days of ingestion of metacercariae, the organism bypasses the peritoneum and reaches the liver parenchyma by entering the Glisson's capsule. While the juvenile fluke moves through the liver, it grows significantly by feeding on host tissue cells and, eventually, on blood (Jefferies et al., 2001). Pathogenesis as a direct result of the fluke activity may be either acute

or chronic, in which acute fasciolosis occurs during the pre-adult migration of flukes in the parenchyma of the liver and chronic fasciolosis is associated with blockage and fibrosis of the bile ducts from feeding adult flukes. Within the mammalian host, the trauma caused by the immature flukes burrowing through the liver parenchyma is associated with most of the pathogenesis. Lalor et al. (2021) thought that this form is rare in cattle. Similarly, the feeding activity and the physical presence of large flukes in the bile ducts can lead to anemia, inflammation, obstruction, and cholangitis (Lalor et al., 2021). *F. gigantica* is more destructive than *F. hepatica* (Sharma et al., 2011) most likely due to the size of the adult flukes.

2.5.6.2 Clinical Signs

The clinical signs that develop depend on the degree of the infection and the stage of the disease. Clinically, sudden death, weakness, pale mucous membranes, difficulty in breathing, jaundice, blood-stained froth from the nostrils, bloody discharge from the anus, abdominal cramps, anemia, colic-like signs, dry feces, and ascites are signs of the invasive phase (acute infection), which frequently results from a high intake of metacercariae over a short period. Sub-acute disease, is often associated with prolonged intake of moderate numbers of metacercariae with symptoms such as anorexia, weight loss, icterus, and sometimes death (Love and Hutchinson, 2007). Depressed growth and milk production are symptoms of chronic disease caused by the consumption of smaller amounts of metacercariae over a longer time, which involves the development of mature flukes in the bile ducts (Radostits et al., 2007). The chronic variant might be asymptomatic in mild infections and is more frequently observed in domestic ruminants. Constipation and diarrhoea in advanced stages are signs of infection in cattle and are frequently present. Ogunrinade and Ogunrinade (1980) reported that fasciolosis can be a precipitating factor to other diseases of livestock, such as infectious necrotic hepatitis and salmonellosis.

2.5.6.3 Pathology

Gross pathology

The amount of metacercariae consumed at one time, the stage of parasitic development in the liver, and the species of *Fasciola* involved all affect the harmful effects that the parasites induce (Lalor et al., 2021). The bile duct-invading adult worm and the invasive stages of *Fasciola* in the liver are both responsible for the effect (Lalor *et al*., 2021). The pathological lesions occur mostly in the liver parenchyma and bile ducts. The adult flukes' hematophagous behaviour and cuticular spines' destruction of the bile duct mucosa occur in the second phase, which follows the first phase's blood loss and liver injury. Though there is a paucity of information on the pathogenesis of *F. gigantica,* it was reported in the literature that only the chronic form of *F. gigantica* disease occurs in cattle, whereas both the acute and chronic forms occur with both species in the sheep (Mochankana and Robertson, 2016).

Clinical Pathology

The extent of the pathological lesion depends on the size of the infecting metacercariae or individual fluke in the bile duct (Fraser, 1991)Rarely does the parasite infect extrahepatic tissues like the brain and eye; instead, it typically infects the liver parenchyma and causes lesions (Zhou *et al*., 2008). The general findings in fasciolosis include anaemia, hypoalbuminemia, and eosinophilia. Lotfollahzadeh *et al*. (2008) estimated blood losses of 0.2 to 0.5 ml per day as the effect of each liver fluke. This implies loss of about half a liter of blood within a period of one week in moderate infection of 100 to 200 flukes in cattle. The main cause of anemia is uncertain, but many reasons have been adjudged for this. For example, Mochankana (2014) reportedly linked anaemia to haemorrhages as a result of unintentional injury to hepatic arteries during the parasite's migratory phase. Lotfollahzadeh *et al*. (2008)

associated the cause of the anaemia with the leakage of red blood cells into the gastrointestinal tract and blood loss into the bile ducts. However, the animal's erythropoietic capacity, which is influenced by the amounts of iron and protein in the food, has a major role in the severity of the anemia rather than the quantity of biliary haemorrhage. In some species, there may be indications of erosion and papillary and glandular hyperplasia of the biliary epithelium. In the chronic lesion, there is evidence of widespread macrophages, epithelioid cells, and multinucleated giant cells, especially around deceased larvae, and the tracts look paler than the nearby parenchyma (Zachary and McGavin, 2013). Additionally, there may be several 0.5 to 5 mm red patches that are uneven when cut, hard and oedematous, along with bleeding in the bile ducts and parenchymal fibrosis (Quevedo *et al*., 2018). The main macroscopic changes observed in chronic fasciolosis are pale liver with the size of the left lobe generally reduced, fibrosis, and a bile duct wall thickened.

2.5.7 Immune responses and immunity

The pattern of the immune response elicited during natural infection with *F. hepatica* in ruminants has been identified, and additional research on *F. gigantica* that reports comparable results is now being conducted (McManus and Dalton, 2006). Acute fasciolosis in cattle exhibits a mixed immune response with increased Interleukin-10, Transforming growth factor-beta, Interleukin -4, and Interferon-gamma, but with the progression of the disease, immune responses become more dominant of T helper 2/Treg. Treg cells release cytokines that inhibit inflammatory Th1/Th2 cytokines during the latter chronic stage of the disease. Since sheep infected with *F. hepatica* likewise exhibit a mixed Th1/Th2 cytokine profile in the spleen at week 3 post-infection and exhibit increased gene expression of Th2 but not Th1 cytokines as the illness develops, this immune response pattern is comparable to that in sheep (Cwiklinski et al., 2016).

2.5.8 Diagnostic approaches

Helminth infections are regularly diagnosed through faecal investigations. *Fasciola* infection in cattle is often asymptomatic, and there are many indications that could be caused by other diseases that are related. Techniques specifically designed to find *Fasciola* eggs in faeces have been developed. Serological methods are also available, and those that find the antigen in the faeces of infected animals can diagnose the fluke earlier and with more accuracy, although they are often only employed in scientific studies.

2.5.8.1 Floatation/ sedimentation techniques

Though flotation technique is been used qualitatively in the detection of nematode and cestode eggs basically for preminary surveys in order to classify the parasite groups. The conventional test for liver fluke that has been in use for a long time is the faecal egg count through sedimentation (Urquhart et al., 1996). This method employs solutions of lower specific gravity than the parasitic organisms, thus allowing for the concentration of the parasitic eggs in the sediment. The method has the benefits of being simple to use, easy to collect samples from, and easy to identify eggs. This method has a high specificity, detects current infection and can be used for ante mortem diagnosis of liver fluke infection (Charlier et al., 2014). The method's drawbacks include limited sensitivity, which will lead to a significant number of false negative results, but repeated analysis and increasing the faecal volume can increase sensitivity up to 90 % (Rapsch et al. 2006). Since the test detects eggs during patent infections, in prepatent infections, organisms will be present but not yet producing eggs, hence producing false negative results. Eggs can only appear in faeces at 10 weeks post-infection and the effects of the disease would have occurred 3 weeks post-infection. It is also difficult to differentiate between the eggs of the two *Fasciola* species (Fagbemi and Obarisiagbon, 1990). Though the sedimentation test cannot differentiate between *Fasciola* species in areas where they overlap,

it remains an important screening test and is widely employed in on farm epidemiological studies (Rojas et al., 2014).

2.5.8.2 Immunological methods

Serum antibody ELISA (AbELISA)

The ability to identify anti-*Fasciola* antibodies in serum has led to the development of various enzyme-linked immunosorbent assays (ELISAs) test types. While these have the advantage of detecting infection very early (4-8 weeks post-infection) (Valero et al., 2009), the antibody titers can persist at high levels following successful treatment (Sánchez -Andrade et al., 2001). Another problem is the anti-*Fasciola* antibodies' serological cross-reactivity with antigens from related trematodes, cestodes, and nematodes. The disadvantages of this are the invasiveness of obtaining blood samples that may be less practicable for farmers than those which just require faecal samples; after the first year of life, the test is likewise only accurate as a predictor of prior exposure and unreliable as a predictor of current infection status, making it less relevant for epidemiological studies.

Serum antigen ELISA

Langley and Hillyer (1989) were the first to disclose the use of sheep to detect *F. hepatica* in mouse serum as opposed to antibodies to *Fasciola* in serum by ELISA. Rodrguez-Pérez and Hillyer (1995) followed suit. This has the advantage of being able to reveal a persistent infection. The test also allows very early detection of infection; 1-2 weeks post-infection. However, as the host mounts an immune response, antibodies interact with the antigen's epitopes to form an immune complex, which reduces the antibody's ability to bind to the antigen in the ELISA (Valero et al., 2009). As a result, this test's sensitivity was weaker when used in the field. Various times have been recorded in the literature for the amount of free circulating antigen to go below detectable levels, while some authors reported it to be absent

from 5 weeks post-infection, others reported it can still be detectable until 10 weeks post-infection (Attallah et al., 2013)

Coproantigen ELISA (coproAg ELISA)

The coproAg ELISA test has the benefit of being simple to perform for herd monitoring at a preliminary fasciolosis stage (Salimi-Bejestani et al., 2005). The coproAg ELISA test, in particular, has great specificity as demonstrated by post-mortem analysis, and high sensitivity as demonstrated by the absence of cross-reaction with antigens from other helminths. It is based on the identification of excretory/secretory (ES) antigens in faeces. Additionally, it provides results within the first 1 to 5 weeks after parasites enter the biliary systems (Valero *et al*., 2009). As mentioned earlier, concerning ease of sample collection, faecal samples are the easiest practicable procedure. As a result, numerous studies have used different capture antibodies to observe parasite antigens in host faeces.

A monoclonal antibody ELISA was found by Abdel-Rahman et al. (1999) to be 100 % sensitive and 90 % specific in cattle post-mortem infections of more than 10 fluke, suggesting infection at 6 weeks post-infection and revealing no cross-reaction with *Paramphistomum microbothroides*, a trematode parasite of livestock. In the same study, a relationship between ELISA titers and parasite burden was also noted and reported. A commercial coproAg ELISA, (BIO K201, Bio-X Diagnostics), based upon the MM3 monoclonal ELISA was developed to find parasite antigens in faeces (Mezo *et al*., 2004). The test has so far been developed to detect *F. hepatica* antigens. It is unknown if it will be effective to detect *F. gigantica* antigens. When diagnosing samples containing >10 EPG and utilizing the manufacturers' recommended positive threshold, numerous investigations have shown that the sensitivity of the coproELISA technology has declined since it was commercialized. However, a more sensitive molecular diagnostic approach for *Fasciola* species-specific has been created, and this has clearly

improved the ability to detect *Fasciola* spp.. This innovative method offers a straightforward, step-by-step procedure for faecal sample preparation, enabling medium-high throughput for the diagnosis of fascioliosis. However, this method's application is only partially useful in places where the diagnostic capability may be constrained or if the required laboratory equipment is not there. When performing an epidemiological survey in rural areas, the capacity to maintain samples in 70 % ethanol for transfer to locations with improved diagnostic capacity is crucial and may be difficult (Calvani *et al*., 2017).

2.5.8.3 Post-mortem liver examination

Another commonly adopted method of diagnosis is the examination of livers during post-mortem inspection. This is done frequently during slaughtering at the abattoir to check on the liver's health and to determine the reason for death. It involves cutting and opening the liver to check for parasites or to see the lesion brought on by a past or present infection. This can be used as a surveillance tool for investigations on prevalence (Froyd, 1975). However, it must be known, that some other reasons may lead to parenchyma tissue abnormalities vis-a-vis liver condemnation. Though this finding may be beneficial to the farmers to change their fluke or worm management or control practices via knowledge of current or historic infection, the economic losses would have been done for that year. In abattoirs, if the inspection is properly performed by trained and experienced personnel, the simplicity of the macroscopic diagnostics ensures the accuracy of liver condemnation rates in slaughtered animals (Alula et al., 2013; Quevedo et al., 2018). Despite its use as a gold standard for diagnosis of fasciolosis, this method is not feasible for ante mortem diagnosis and on-farm epidemiological studies.

2.5.8.4 Haematology and biochemistry

The diagnosis of subacute and chronic liver fluke infection can be made using changes in the values of several haematological and biochemical indices. Gamma-glutamyl transferase

(GGT), glucose concentrations, globulins, segmented and band neutrophil counts, mean corpuscular volumes, and white blood cell counts were all shown to be elevated in sheep with *F. hepatica* infections (Matanović *et al*., 2007). In the same study, it was found that infected animals had lower levels of some parameters than uninfected animals, including haemoglobin, lymphocytes, red blood cell counts, packed cell volumes, mean corpuscular hemoglobulin concentrations, aspartate aminotransferase, blood urea nitrogen, creatinine, and albumins. Defects associated with the bile duct and liver can be established through assessment of the level of GGT and glutamate dehydrogenase (GLDH) values respectively. Assessment of GLDH level has been used as an indicator of infection as early as 2-3 weeks post-infection (Mitchell, 2002). However, the adoption of this as a diagnostic indicator may not be absolute due to the potential for false positives, and thus misdiagnosis, as many other underlying causes of similar clinical symptoms have been reported. Also, there is a disadvantage of invasiveness as a blood sample is required to be taken.

2.5.8.5 DNA-based methods

It is challenging to employ methods like the body length-to-width ratio for morphological characterization and differentiation of *Fasciola* species due to the differences in their sizes, especially with respect to the fluke's age, host species involved, and fixation technique adopted (Ichikawa and Itagaki, 2012). Due to limitations of the morphological technique, several molecular strategies have been developed that use various molecular targets to distinguish between *F. hepatica* and *F. gigantica* (Huang *et al*., 2004). These DNA-based detection methods are not yet fully in use for herd screening for *Fasciola* infection. Their cost is still expensive for farmers to afford and most of the techniques are still underdeveloped in their current status. Despite this, DNA-based detection methods can accurately and quickly identify

infections. The following are the DNA-based methods that have been developed and used for years.

2.5.8.5.1 Polymerase chain reaction (PCR)

PCR is a widely used technique for DNA amplification. Using opposing primer pairs, Taqman polymerase, a specialized buffer, and dNTPs, heat cycling is used in PCR to induce exponential amplification of a DNA sequence. Successful PCR amplification of DNA isolated from adult *F. hepatica* parasites and infected snails has been carried out (Ai *et al*., 2011). These authors demonstrated the possibility of quantifying the quantity of DNA amplified using real-time PCR.

Few reports exist of the effective amplification of DNA isolated from animals infected with *F. hepatica*. The identical primer set was successful in amplifying DNA isolated from faecal samples from infected animals two weeks after infection, according to (Robles-Perez et al., 2013). Despite the high sensitivity and specificity potential of faecal-based diagnosis using PCR, the assay has limitations. Such as the inhibition of Taq polymerase by the substances, which may hamper the sensitivity of the test (Wilson, 1997). Additionally, this test required the use of agarose gel electrophoresis and thermocycler for PCR procedures. Furthermore, higher potential for sample contamination that may affect specificity, if precautionary measures were not taken. Lastly, issues related to high cost and more time-consuming very lengthy steps involves DNA extraction before PCR. Due to these restrictions, the test must be performed under sterile laboratory conditions, which raises the cost of the assay. For most farmers, the cost of this test can be a considerable worry.

2.5.8.5.2 The real-time PCR

Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity, its presently a known method in clinical and veterinary diagnostics and food safety sector since organism quantification and typing is possible with qPCR. (Kralik and Ricchi *et al*., 2017). Pathogen detection is made possible through the amplification of a specific fragments of DNA. qPCR assay designs for every or most organisms become possible in recent times due to the availability of sequencing data. The technique has a greater advantage in providing quick and high-throughput establishment and evaluation of target DNA sequences in different matrices. The rapid period of amplification is possible through simultaneous amplification and visualization of newly formed DNA amplicons. It is also safer regarding contamination as no subsequent sample manipulation is required post-amplification. It also has a wider dynamic range for quantification (7-8 Log_{10}) and conversion of several amplification into a single reaction (Klein, 2002). Detection of specific genes and alleles is possible with qPCR, but caution must be included to know that mere detection of genes does not automatically result in those particular genes' production or expression. Therefore, as fast and advantageous as the test seems to be, the results must often compare with other phenotypic detection methods (Osei Sekyere et al., 2015)

2.5.8.5.3 The Loop-Mediated Isothermal Amplification method (LAMP)

In addition to being used to diagnose infectious diseases, the Loop-Mediated Isothermal Amplification technique (LAMP) has also been used to identify and characterize harmful organisms. This method has the benefits of isothermal multiplication (between 59 and 66 degrees Celsius) and the ability to start the detection process with a tiny amount of DNA.

inexpensive, simple to use, and easily accessible approach. High specificity since the target sequence's 6 gene sections can be detected by its 4 primers at the start of the reaction, and 4 additional regions can be detected during the LAMP reaction process. It only needs a primer, DNA polymerase, and reaction mixture; it is also possible to perform it without a thermocycler using a heated water bath or thermal blocks, which has several other benefits. These are this technique's drawbacks. Lack of commercial kits based on the LAMP technique, the complexity of various primers' designs for multiplication of new gene areas and choosing the appropriate locations in the gene sequence for efficient construction of primers, and a complicated product featuring cauliflower-like structures with varying sizes. The popularity of this technology is hampered by some of its drawbacks, including the intricacy of its mechanism, which makes it less popular than PCR-based methods. However, the LAMP approach has several advantages and, with some improvements, might be a significant alternative to the PCR method (Keikha, 2018).

2.5.9 Production, economic and zoonotic importance

2.5.9.1 Production Impact

Fasciolosis infection in ruminants generally presents poor body condition and overall lowered productivity. Due to production losses, the disease is a significant barrier to effective domestic ruminant production, particularly in tropical nations (Mage et al., 2002). Subclinical infections also cause decreased feed efficiency, growth retardation, decreased milk production, poor reproductive performance, poor carcass quality, decreased work output in draught animals, increased susceptibility to other diseases, and high liver condemnation. The disease also has a negative impact on the health of the liver. Due to appetite suppression and its impact on the post-absorptive metabolism of protein, carbohydrates, and minerals, parasites can significantly

affect output (Shea-Donohue *et al*., 2007). In most cases, chronic fasciolosis infections always result in poor body conditions of the affected animals (Zachary and McGavin, 2013).

2.5.9.2 Economic Impact

Fasciola infection is one of the most important endo-parasitic diseases of domestic and wild ruminants globally, resulting in great economic losses, which may be due to liver condemnation, mortality, production losses associated with milk and meat, secondary bacterial infections, reproductive failure, and the cost of frequent anthelmintic treatments (Beesley *et al*., 2018). The direct losses due to fasciolosis include; animal mortality and organs condemnation at abattoirs, while the indirect losses may include; reduction in carcass weight, delayed growth rate, reproductive failure, infertility, reduction in milk yield, cost of treatment expenses, declined production and productive performances and animals being predisposed to other diseases (Le *et al*., 2007). With over 600 million infected animals, these economic damages to the worldwide agricultural community have been estimated at nearly 3.2 billion US dollars annually (Mas-Coma *et al*., 2005).

The economic losses in tropical countries have been estimated to be able to have reached more than 3200 million US dollars from 1975 – 1997 (Hamoo *et al*., 2019). The species infect the bile duct and liver of cattle, buffaloes, sheep, goats, and pigs, which has a significant consequence on the growth rate, development, and productivity in general (Kuchai *et al*., 2011). According to estimates by Abebe et al. (2010), the cost of liver condemnation in Sudan is estimated to be US$1.94 million for both cattle and sheep, while the cost of liver condemnation in Uganda is estimated to be US$92,474,620 each year (Joan et al., 2015). Within four months, the condemnable weight of 342kg of livers from just 38 calves with fasciolosis was valued at N136,800.00 in Akwa Ibom State, Nigeria (Usip et al., 2014). Over 700 million Birr dollars were lost annually in Ethiopia as a result of parasites, particularly

fasciolosis (Yimam, 2003). In three abattoirs in South Africa's Eastern Cape Province, Fasciola infection resulted in a total financial loss of ZAR 44, 930 (3456.2 USD). Partial liver condemnation was predicted to cost ZAR 19, 700, while entire liver condemnation was valued at ZAR 25, 230 (2, 357 USD) (Jaja *et al*., 2017).

Figure 2.5. Numerous adult flukes of *Fasciola hepatica* in the bile ducts and liver parenchyma of an infected cow (Lalor etr al., 2021)

2.5.9.3 Zoonotic Importance

Due to its zoonotic potential, fasciolosis has been acknowledged as a disease of public health relevance (Thanh, 2012). However, reports of the sickness in humans are only a few (Hillyer, 1999), and it has long been less prioritized (Mas-Coma et al., 2009). As a result of current epidemiological data, the disease has recently come to be recognized as an important rising public health problem of serious concern. Infection in people occurs by ingesting aquatic plants that contain infected metacercariae, eating vegetables contaminated with metacercariae, or

even drinking untreated water polluted with metacercariae. The disease is a significant plant- or food-borne trematode zoonoses. Raw livers from sick sheep, goats, or cattle have been linked to human illness in some cases (Soliman, 2008).

2.5.10 Prevention and control of fasciolosis

Anthelmintic therapy is the only known treatment for clinical fascioliosis and the specific drug of choice is Triclabendazole, a member of the benzimidazole group. Administration of anti-fluke may be commenced from September to late January at 10 weeks intervals in moderate fluke-infested areas. Treatment may be required from April to November at 10 weeks intervals in areas with high fluke risk. Other methods of control include bush clearing to reduce potential snail habitats and limit the exposure of animals to infected grazing areas. Zero-grazing systems minimize the incidence of fasciolosis in animals, and this is done to disrupt the life cycle of the fluke (Coyne et al., 2020). There have been arguments on the use of molluscicides to control fluke; the negative effect of molluscicides on the ecology of wildlife has been stated as the reason. There have also been rising reports of failure of both mollscicides and flukicides. Avoiding snails, the invertebrate host, rather than eliminating them is now a part of the fluke management measures (Sargison and Scott, 2011). Fencing off of water-logged areas which are suitable habitats for the snail intermediate host may not be practicable as a large expanse of pasture land would require to be fenced off for this to be achieved.

2.5.11 Overview of knowledge, and practices about keys epidemiological factors of bovine fasciolosis

Ample and in-depth knowledge about the views and daily routines of interventions receives are of utmost importance for the efficient prevention and control of any diseases, whether in cattle or humans (Bhopal, 2016). Effective disease prevention and control may be hampered by factors such as inadequate education or illiteracy, lack of political will, poverty, impassable

roads and neighbourhoods, inadequate disease evaluation efforts, and a lack of infrastructure that allows for frequent treatment (Larrieu and Zanini, 2012). In an endemic hamlet in Leyte, the Philippines, Francisco et al. (2019) adopted the used of questionnaire surveyas a method to assess knowledge, attitudes, and practices on schistosomiasis transmission, symptoms, behaviour, and control. Communal farmers' knowledge, attitudes and practices about bovine fasciolosis in the North West Province of South Africa, have not been documented, and thus, require investigation.

2.6 Summary

As important as bovine fasciolosis is, there is little or no epidemiological information on the disease in the communal farming areas of the North West province of South Africa. It will be of great necessity to establish the current status of bovine fasciolosis in the communal farming operation of the province under the following objectives: determination of farmers' knowledge, attitude and practices, comparison of different detection methods in establishing fasciolosis prevalence, establishment and comparison of haematological and biochemical parameters in infected and non-infected cattle in the study areas and molecular detection of *Fasciola* species in the study areas through sequencing and phylogenetic analyses.

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Chapter 3: The knowledge, attitudes and practices of smallholder cattle farmers concerning the epidemiology of bovine fasciolosis in the North West Province, South Africa

(Under review in Tropical Animal Health and Production)

Abstract

Bovine fasciolosis has negative impacts on cattle production worldwide, more so on the African continent and especially in smallholder farming areas with a limited level of awareness. A cross-sectional questionnaire-based survey was conducted to investigate the knowledge, attitudes, and practices concerning bovine fasciolosis among smallholder cattle farmers in the North West province of South Africa. A total of 153 farmers were interviewed from three villages of the Moretele Local Municipality in Bojanala District. Majority of respondents were male (84 %) and (81 %) had low education levels (56 % primary school or less) and employed extensive cattle management systems (84%). A large number of farms lacked infrastructure including calving pens (88 %), restraining equipment (85 %), and weight-determination equipment (92 %) with rivers or dams (58 %) as sources of drinking water for cattle. No factors were associated with a positive fasciolosis epidemiological knowledge score. However, level of education ($P = 0.046$), some cattle breeds ($P = 0.022$), and management system ($P < 0.001$) of the smallholder farmers were associated with a positive practice score concerning bovine fasciolosis prevention.

We, therefore, recommend a public enlightenment on the mode of transmission, risk factors, zoonotic importance, and practices associated with the prevention and control of bovine fasciolosis.

Key words: beef production, epidemiology, liver fluke, perceptions

3.1 Introduction

Many rural communities in Africa make their livelihoods from cattle production, which also provides essential dietary components of milk and meat (Kabubo-Mariara, 2009). The livestock sector contributes more than 40% to the gross domestic product (GDP) of South Africa's agricultural economy (Masemola et al. 2019). Smallholder cattle farmers are defined as poorly-resourced farmers with small plots for the rearing of cattle both for household food and for

nutritional security (Udo et al. 2011), and who depend on this sector for their livelihoods (Rootman et al. 2015). The roles of cattle for smallholder farmers include sociocultural (traditional ceremonies, sacrifice purposes), economic (family financial base, property protection, livelihood), and sustainable agricultural production purposes (traction for tillage, manure as fertiliser for crops, agricultural diversification) (Ndoro et al. 2014). Notwithstanding these benefits, smallholder cattle production is constrained by several factors, chief which is parasitic diseases. Fasciolosis (liver fluke infection) is considered the most important parasitic disease and a major impediment to sustainable cattle production, especially in the tropical region (Bayer et al. 2003).

Liver fluke infection (fasciolosis) is a neglected tropical disease (parasitic zoonosis) of animals and people (fasciolosis). Infestation with *Fasciola hepatica* and *Fasciola gigantica* liver flukes are the cause of the disease and intermediate snail hosts are required for the pre-parasitic developmental phase of these parasites (Lalor *et al*., 2021). Factors including climactic conditions (adequate moisture and temperature) and the presence of definitive mammalian hosts are essential for the completion of the parasite's life cycle (Fairweather, 2011). The importance of host attributes (sex, age, and breed) and seasonal risk factors for fasciolosis in domestic ruminants have been previously described (Islam et al. 2014). The roles played by vegetation and water plant species in the transmission of fasciolosis, especially human fascioliasis have long been established in most developed countries (Mas-Coma et al. 2018). In cattle, fasciolosis causes anaemia and hypoproteinaemia, which contribute to herd morbidity and mortality. Additional effects on cattle production include reduced milk yield, poor growth, and reproductive performance, and increased production costs due to required treatments (Beesley et al. 2018). Management of fasciolosis is by anthelmintic therapy, specifically triclabendazole, a member of the benzimidazole group (Merachew and Alemneh, 2020).

Fasciolosis has been reported to have a higher prevalence in cattle herds reared by smallholder farmers due to high illiteracy rates, poor recognition of the disease, limited resources for control, suboptimal nutrition, and poor biosecurity (Nyindo and Lukambagire, 2015). Poor off-take and reduced incomes are characteristic of smallholder cattle operations (Molefi and Mbajiorgu, 2017). It is essential to control bovine fasciolosis in smallholder cattle herds, and attention should be given to the farmers' perceptions and practices concerning the disease, as these will affect the success of implemented control measures.

Studies on knowledge, attitudes and practices (KAP) of smallholder farmers on bovine fasciolosis have been used to assess their willingness to adopt prevention and control measures (Tiongco et al. 2012). Inadequate knowledge of the disease, the presence of multiple high-risk farm practices, inappropriate perceptions, and bad practices require education for improvement. Assessment of farmers' KAP on bovine fasciolosis is essential for the development of appropriate policies and strategies to prevent and control the disease (Aregahagn and Melkamu, 2018). The current study was, therefore, aimed at assessing smallholder cattle farmers' knowledge and awareness of risk factors, zoonotic importance, transmission, prevention and control of bovine fasciolosis in the North-West Province of South Africa.

3.2 Materials and Methods

3.2.1 Description of the study site

The study was conducted in three villages (Mkapanstad, Ga-Motle and Tladistad) in the Moretele Local Municipality, falling under the Bojanala District Municipality in the North West Province of South Africa (Figure 1). Makapanstad is located at 25° 14' 36" South and 28° 7' 19" East and has a total area of 20.45 km^2 and a human population of 15000. Ga-Motle is located at 25° 21' 14" South and 28° 4' 9" East and encompasses an area of 8.3 km^2 with a

human population of 5600. Tladistad is located at 25° 12′ 10.8″ South and 28° 2′ 6″ East, with an area of 3.30 km^2 and a human population of 3000 (Letsoalo et al. 2000). A brief description of the area should be appropriate, concerning the predominant occupation of the people, the vegetation and climate, etc

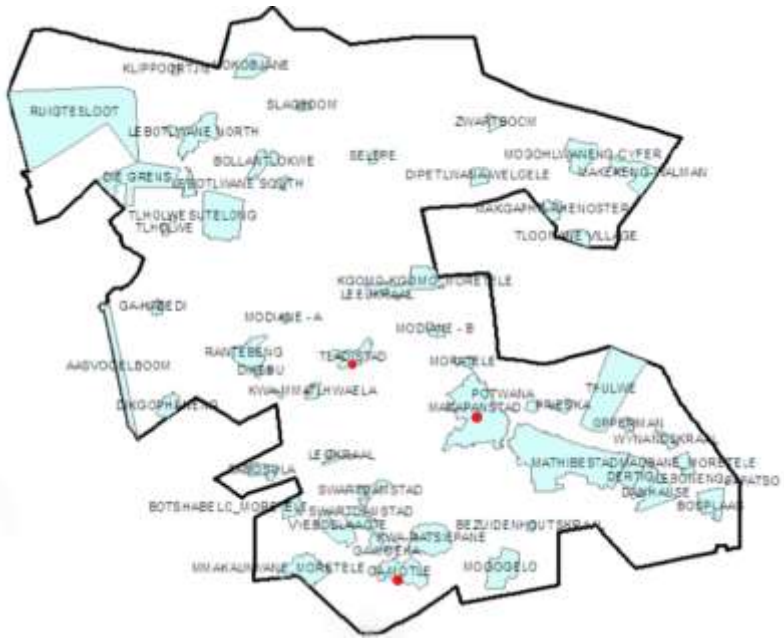

Figure 3.1: Map of Moratele Local Municipality showing the three study sites marked in red. Adapted from Maime (2015)

3.2.2 Farmer selection and data collection

The district and local municipality were selected based on the willingness of farmers to participate in the study, the availability of cattle, and the presence of semi-intensive and extensive smallholder cattle farmers in the area. Smallholder farmers were selected using the snowball sampling technique (Qokweni et al. 2020). Inclusion criteria were active smallholder cattle farmers owning more than four cattle, which consented to participate, and were at least 18 years-old. A paper-based mixed ended questionnaire was pre-tested and then administered

to a total of 154 farmers in the villages of Makapanstad (n=62), GaMotle (n=41), and Tladistad (n=50). Informed consent was obtained before the interviews and respondents were assured that their identity and responses would not be disclosed. The questionnaire involved four major sections (A, B, C. and D) with sub-sections containing 39 major questions and several questions under each major question. The estimated time of completion of the questionnaire was 30 minutes. Section A requested information on farmers' demographic information, and section B requested herd structure demography information. Section C requested information on farmers' level of knowledge/awareness on clinical signs, mode of transmission, zoonotic importance, and risk factors of bovine fasciolosis. Section D requested information on practices associated with the prevention and control of bovine fasciolosis.

3.2.3 Statistical analyses

Data were entered into a Microsoft Excel® (Microsoft Corporation, USA) spreadsheet and then analysed using the Statistical Package for Social Scientists (SPSS Version 26). Descriptive statistics were used to present data on farmer and herd demography, farm characteristics, and management practices. The association between location and farmer and herd demographic, farm structure and management variables were determined using chi-square tests. The epidemiology knowledge and practice scores were established as follows: correct responses were scored as +1, incorrect responses as -1 and unsure as 0. These were inputted on the excel spread sheet and to summed up as total scores concerning epidemiological knowledge (questionnaire section C) and beneficial fasciolosis practices (questionnaire section D). Total scores greater than 0 were considered indicative of positive for epidemiological knowledge and fasciolosis practices. Binary logistic regression was used to investigate the association of potential predictors and having positive knowledge and practices independently. Univariate screening models were fit and all predictors with Wald $P < 0.2$ were selected for multivariable

modeling. Multivariable models were fit using a manual backward elimination process starting with all variables identified in the univariate screening models. Variables were removed one-by-one based on the largest Wald P value until all remaining variables were $P < 0.05$. The fit of the final model was assessed using a Hosmer-Lemeshow test. Odds ratios (OR) and p-values were used to estimate the level of association and statistical significance, respectively. Odd ratios were calculated with 95% confidence intervals (CI) and $P < 0.05$ was used to determine statistical significance.

3.3 Results

3.3.1 Farmer and herd demographic information and farm infrastructure

Majority of the farmers were males (129/153) and most of the respondents owned their farms (124/153) versus being hired herdsmen (29/153). A larger proportion of the farmers had no formal education or completed only primary education (86/153). Most of the farmers are married (120/153) and the majority had more than 10 years of cattle-rearing experience (125/153). A large proportion of farmers practiced extensive cattle management (128/153).

Non-descript or crossbred cattle (94/153) were most common followed by Brahman (42/153) and lastly Bonsmara (17/153). Farmers reported the body condition score of their cattle to be mostly average (69/153), followed by poor (63/153), and lastly good (21/153). Most farmers solely grazed their cattle on pastures (109/153) while fewer included feed supplements with pasture grazing (44/153). Many farms lacked infrastructure including calving pens (134/153), restraining equipment (130/153), and weight determination equipment (140/153). Most farmers (89/153) sourced drinking water from streams/rivers or dams followed by wells (24/153) and municipality water (16/153) in that order. Sixteen percent (24/153) of farmers used more than one water source.

Farmer cattle management system significantly varied among the study village (P<0.001) but none of the other evaluated demographic variables varied significantly (P > 0.05; Table 1). Ga-Motle (11/41) and Tladistad (11/51) had higher proportions of semi-intensive farmers compared to Makapanstad (3/62). The source of drinking water varied among the study villages (P < 0.001) but none of the other evaluated categorical predictors differed by location (P > 0.05; Table 2). Dam or river water was the most common source across all locations followed by well water in Makapanstad, as opposed to multiple water sources in GaMotle and Tladistad.

3.3.2 Predictors of positive epidemiological knowledge and practices

No variables were significantly associated with positive epidemiological knowledge of fasciolosis (Table 3). However, there was significant association between positive practice scores and some categorical predictors. Farmers in Tladistad Village Thad higher likelihood of a positive practice score compared to Kroimkain, Village K while male farmers and farmers' practicing an extensive system of management had a significantly lower likelihood of positive practice scores compared to female farmers and farmers with a semi-intensive system of management, respectively (Table 4).

Multivariable modeling identified education level (P = 0.046), cattle breed (P= 0.022), and farmers' system of management (P=0.001) as independent predictors of positive practice scores concerning bovine fasciolosis prevention (Table 5). The final model was an adequate fit to the data based on the results of the Hosmer and Lemeshow test (χ^2 = 2.686, df = 4, P = 0.612).

Table 3.1: The association between location and potential categorical predictors of 153 smallholder cattle farmers in communal areas of North West Province South Africa from June to Oct 2019.

Variables	Total (n=153)	Village M (n=62) Frequency	%(95%CI)	Village T (n=41) Frequency	%(95%CI)	Village K(n=50) Frequency	%(95%CI)	P<value*
Farm ownership								
Owner	124	47	76(64-85)	33	80(66-90)	44	88(76-94)	0.261
Hired hand	29	15	24(15-36)	8	20(10-34)	6	12(7-24)	
Sex								
Male	129	51	82(71-90)	34	83(69-91)	44	88(76-94)	0.680
Female	24	11	18(10-29)	7	17(9-31)	6	12(7-24)	
Marital status								
Single	15	9	15(8-25)	3	7(3-19)	3	6(2-16)	0.139
Married	120	50	81(69-89)	32	78(63-88)	38	76(63-86)	
Divorced/widow	18	3	5(2-13)	6	15(9-28)	9	18(10-31)	
Education level								
No formal education	23	9	15(8-25)	4	10(4-23)	10	20(11-33)	0.388
Primary	63	22	35(25-48)	18	44(30-59)	23	46(33-60)	
Secondary/tertiary	67	31	50(38-62)	19	46(32-61)	17	34(22-48)	
Language								
Sepedi	59	29	47(35-59)	10	24(14-39)	20	40(28-54)	0.147
Xhosa	12	5	8(3-18)	5	12(5-26)	2	4(1-13)	
Tswana	82	28	45(33-57)	26	63(48-76)	28	56(42-69)	
Management system								
Backyard	44	31	50(38-62)	4	10(4-23)	9	18(10-30)	<0.001

Extensive	84	28	45(33-57)	26	63(48-76)	30	60(46-72)	
Semi-intensive	25	3	5(2-13)	11	27(16-42)	11	22(13-35)	
Farming experience								
Less than 10 years	28	16	26(17-38)	7	17(9-31)	5	10(4-21)	0.158
10 to 20 years	76	29	47(35-59)	23	56(41-70)	24	48(35-61)	
More than 20 years	49	17	27(18-40)	11	27(16-42)	21	42(29-56)	

Table 3.2: The association between locations and potential categorical predictors in the herd structure of smallholder cattle farmers in communal areas of North West Province South Africa from June to Oct 2019.

Variables	Total	Village M (n=62) Frequency	%(95%CI)	Village T (n=41) Frequency	%(95%CI)	Village K (n=50) Frequency	%(95%CI)	P-value*
Herd structure								
single	150	61	98(91-100)	40	98(87-100)	49	98(90-100)	0.957
multiple	3	1	2(0.2-9)	1	2(0.4-13)	1	2(0.4-10)	
Cattle breed								
Brahman	42	15	24(15-36)	15	37(24-52)	12	24(14-37)	0.133
Bonsmara	17	9	15(8-25)	6	15(7-28)	2	4(1-13)	
Nondescript	94	38	61(49-72)	20	49(34-64)	36	72(58-83)	
Body condition score								
poor	63	18	29(19-41)	21	51(36-66)	24	48(35-61)	0.099
average	69	34	55(43-67)	17	41(28-57)	18	36(24-50)	
good	21	10	16(9-27)	3	7(3-19)	8	16(8-29)	
Type of feed								
pasture	104	44	71(59-81)	29	71(56-82)	31	62(48-74)	0.544
mixed feed	49	18	29(19-41)	12	29(18-44)	19	38(26-52)	

Drinking water source								
dam/river	89	30	48(36-61)	24	59(43-72)	33	66(52-78)	<0.001
municipal water	16	9	15(8-25)	6	15(7-28)	3	6(2-16)	
well	24	20	32(22-45)	2	5(1-16)	2	4(1-13)	
multiple	24	3	5(2-13)	9	22(12-37)	12	24(14-37)	
Calving pen								
yes	19	8	13(7-23)	3	7(3-19)	8	16(8-29)	0.453
no	134	54	87(77-93)	38	93(81-97)	42	84(71-92)	
Restraining equipment								
yes	23	8	13(7-23)	7	17(9-31)	8	16(8-29)	0.823
no	130	54	87(77-93)	34	83(69-91)	42	84(71-92)	
Weighing equipment								
yes	13	5	8(3-18)	1	2(0.4-13)	7	14(7-26)	0.142
no	140	57	92(82-97)	40	98(87-100)	43	86(74-93)	

Table 3.3: Univariate associations between a positive epidemiological knowledge score (score > 0 yes versus score < 0 no) and potential covariates of smallholder cattle farmers in communal areas of North West Province South Africa from June to Oct 2019.

Variable	Level	Parameter estimate ($\hat{\beta}$)	Odds ratio (95% CI)	P value
Location	Village M	0.304	1.36 (0.59, 3.10)	0.471
	Village T	0.043	1.04 (0.41, 2.66)	0.929
	Village K	Referent		
Individual	Owner	0.790	2.20 (0.78, 6.20)	0.135
	Attendant	Referent		
Sex	Male	0.225	1.25 (0.46, 3.40)	0.658
	Female	Referent		
Age	< 60 years	-0.177	0.84 (0.33, 2.12)	0.709
	60 – 69 years	0.129	1.14 (0.47, 2.76)	0.776
	≥70 years	Referent		
Marital status	Married	-0.760	0.47 (0.16, 1.36)	0.163
	Widow	-0.624	0.54 (0.13, 2.25)	0.394
	Single or divorced	Referent		
Education	No formal education	Referent		
	Primary	-0.090	0.91 (0.32, 2.59)	0.866
	Secondary or tertiary	-0.100	0.91 (0.32, 2.55)	0.850
Language	Sepedi	0.258	1.29 (0.61, 2.77)	0.506
	Xhosa, Zulu, Afrikaans, or English	0.542	1.72 (0.59, 5.00)	0.320
	Other language	Referent		
Experience	< 10 years	-0.204	0.82 (0.31, 2.18)	0.685
	10 -20 years	-0.701	0.50 (0.23, 1.10)	0.083
	> 20 years	Referent		
Cattle breed	Brahman	0.105	1.11 (0.49, 2.51)	0.800
	Nguni, Bonsmara, or non-descript	-0.148	0.86 (0.34, 2.20)	0.757
	Multiple breeds or other	Referent		
Management	Backyard	-0.036	0.96 (0.32, 2.89)	0.948
	Extensive	0.086	1.09 (0.41, 2.93)	0.865

	Semi-intensive	Referent		
Feed source	Grazing only	-0.132	0.88 (0.42, 1.84)	0.728
	Mixed or concentrate feeding	Referent		

Table 3.4: Univariate associations between a positive practice score (score > 0 yes versus score < 0 no) and potential covariates of smallholder cattle farmers in communal areas of North West Province South Africa from June to Oct 2019.

Variable	Level	Parameter estimate ($\hat{\beta}$)	Odds ratio (95% CI)	P value
Location	Village M	0.338	1.40 (0.65, 3.04)	0.391
	Village T	0.908	2.48 (1.06, 5.80)	0.036
	Village K	Referent		
Individual	Owner	-0.256	0.77 (0.34, 1.74)	0.535
	Attendant	Referent		
Sex	Male	-0.936	0.39 (0.16, 0.96)	0.041
	Female	Referent		
Age	< 60 years	0.318	1.38 (0.59, 3.18)	0.457
	60 – 69 years	0.091	1.10 (0.48, 2.51)	0.830
	≥70 years	Referent		
Marital status	Married	-0.120	0.89 (0.31, 2.54)	0.824
	Widow	0.608	1.84 (0.46, 7.31)	0.388
	Single or divorced	Referent		
Education	No formal education	Referent		
	Primary	-0.112	0.89 (0.34, 2.39)	0.824
	Secondary or tertiary	0.472	1.60 (0.61, 4.21)	0.338
Language	Sepedi	-0.694	0.50 (0.25, 1.01)	0.053
	Xhosa, Zulu, Afrikaans, or English	-0.345	0.71 (0.26, 1.96)	0.506
	Other language	Referent		
Experience	< 10 years	-0.044	0.96 (0.36, 2.52)	0.929
	10 -20 years	0.544	1.72 (0.83, 3.59)	0.147
	> 20 years	Referent		
Cattle breed	Brahman	0.170	1.19 (0.56, 2.53)	0.660
	Nguni, Bonsmara, or non-descript	0.652	1.92 (0.83, 4.44)	0.127
	Multiple breeds or other	Referent		
Management	Backyard	-1.712	0.18 (0.06, 0.54)	0.002
	Extensive	-1.689	0.19 (0.07, 0.51)	0.001

	Semi-intensive	Referent		
Equipment	Has some equipment	0.447	1.56 (0.76, 3.21)	0.224
	No equipment for management	Referent		
Feed source	Grazing only	-0.965	0.38 (0.19, 0.77)	0.007
	Mixed or concentrate feeding	Referent		
Water source	River	-0.591	0.55 (0.29, 1.06)	0.076
	Other source	Referent		

CI = confidence interval.

Table 3.5: Multivariable associations between a positive practice score (score > 0 yes versus no) and potential covariates of smallholder cattle farmers in communal areas of North West Province South Africa from June to Oct 2019.

Variable	Level	Parameter estimate ($\hat{\beta}$)	Odds ratio (95% CI)	P value
Education	Secondary or tertiary	0.724	2.06 (1.01, 4.20)	0.046
	Less education	Referent		
Cattle breed	Nguni, Bonsmara, or non-descript	1.029	2.80 (1.16, 6.77)	0.022
	Other breeds	Referent		
Management	Backyard	-2.174	0.11 (0.03, 0.38)	<0.001
	Extensive	-1.803	0.17 (0.06, 0.47)	<0.001
	Semi-intensive	Referent		

CI = confidence interval.

3.4 Discussion

The current study sought to understand smallholder cattle farmers' level of knowledge, attitudes, and practices on the epidemiology of bovine fasciolosis, which is an important task before embarking on any intervention strategies to control this parasitic disease. Farmers' demographic structure was similar to the findings of Katikati and Fourie (2019) in a study on improving management practices of emerging cattle farmers in selected areas of the Eastern Cape Province of South Africa. The finding that most respondents were older farm owners with more than 10 years of cattle-rearing experience might be due to rural-urban migration where the elderly are left to farm and the more active youth seek employment and educational opportunities in urban areas (Mlambo 2018; Njwambe et al. 2019; Tada et al. 2020). This agrees with Oladele et al. (2013) who also reported a similar trend in the predominance of older and more experienced farmers in selected villages in the same province. With the increase in unemployment levels in South Africa, more career guidance should be given to rural youth to encourage participation in cattle production.

More males were observed to be involved in cattle farming than females in the current study, most likely due to cattle operations often requiring physically demanding work. This is consistent with the findings of Chah et al. (2013) and Idamokoro et al. (2019) who also reported more males than females participating in livestock farming in rural villages of South Africa. The low level of education attained by farmers observed in the present study might be related to smallholder farmers operating in rural settings and do not have opportunities for higher education. This is similar to Yawa et al. (2020) who also reported low levels of education among cattle farmers in communal areas in the Eastern Cape Province of South Africa.

Cattle herd characteristics observed in the present study were typical of a communal livestock setting. The small herd sizes and abundant crossbred cattle might indicate low socio-economic status and lack of basic infrastructure necessary for the survival of improved exotic breeds. This agrees with the reports of Scholtz et al. (2008) who also reported an abundance of crossbred or non-descript cattle in South Africa. The small herd sizes in this study agree with the findings of Mapiye et al. (2009) who reported low cattle numbers per household in a communal farming setting in South Africa. The lack of basic farm equipment in virtually all herds and the reported average to poor body condition score of cattle observed likely indicate poor socio-economic status of the smallholder farmers. These findings agree with the reports of Schwalbach and Marfo (2001) who reported similar lack of farm infrastructure due to farmers' low socioeconomic status in the North West Province of South Africa.

There were no significant associations between independent predictors evaluated and the epidemiological knowledge score concerning bovine fasciolosis among the smallholder cattle farmers studied. This could be due to many similarities between the farmers in the study areas; socio-demographic structure, herd structure, and climatic conditions were similar in all the villages. This finding agrees with that of Deka et al. (2020) who reported no significant association between farmers' location and their knowledge score on zoonotic diseases in India. These findings also corroborate the observations of Çakmur et al. (2015) who also reported no significant difference between farmers' knowledge of zoonotic diseases and most independent predictors in Kars, Turkey.

The lack of significant predictors suggests that the level of knowledge in sampled communities is relatively unpredictable, and that they possessed random level of knowledge. This could also

indicate a general lack of training, and few people with such knowledge in the study area. The general lack of knowledge concerning bovine fasciolosis among smallholder cattle farmers observed in this present study might also be due to inadequate veterinary extension services in the area. Most farmers possessed low educational qualifications, which might limit their exposure and awareness about bovine fasciolosis. Similarly, several studies have reported poor knowledge among farmers in terms of transmission, prevention and control of zoonosis (Çakmur et al. 2015; Hundal et al. 2016; Singh et al. 2019). This result is consistent with previous recommendations that the most effective intervention strategies to increase cattle farmer's knowledge of animal diseases are continuous 'on-the-job' and 'informal training' Nampanya et al. (2012). Munyeme et al. (2010) attributed this low/random level of knowledge to remoteness, low training status on rearing and handling of animals, lack of health facilities, poor extension services, and low literacy rate among cattle farmers.

The association between farmers' positive practice scores and independent predictors is similar to what Çakmur et al. (2015) reported in their work on the assessment of farmers' practices concerning zoonotic diseases in Kars, Turkey. A previous study also reported a positive influence of farmers' educational status, income levels, and size of enterprise on their knowledge, attitude, and practices toward zoonotic diseases (Özlü et al. 2020). Also, Moutos et al. (2022) reported that ruminant farmers' level of education and extent of veterinary supervision were the only independent predictors for their evaluated practice scores in the assessment of knowledge related to zoonotic diseases in Elassona Municipality, Greece. Positive associations were also reported between farmers' age and educational status and increased practice scores related to antibiotics

use and resistance among animal farm owners/workers in Amhara region, north western Ethiopia (Geta and Kibret, 2021).

In this present study, farmers' educational level, system of management ,and cattle breeds were the predictors that were retained in our final multivariable model. There was a higher likelihood that educated farmers will have more positive practices that can help in the prevention and control of bovine fasciolosis. This finding is similar to the observation of Sadiq et al. (2021), who reported that ruminant farmers with higher educational qualifications have better knowledge to implement practices against zoonotic diseases in Selangor, Malaysia. In this present study, smallholder farmers that owned Nguni, Bonsmara, or non-descript breeds of cattle also had a higher likelihood of improved practices about prevention and control of bovine fasciolosis compared to farmers that reared other breeds. The reason that explains this might be associated with a few numbers of Nguni, Bonsmara and Non-descriptive breeds in the study population. It could also be due to long years of rearing experience by the smallholder farmers owing these breeds, as Nguni, Bonsmara and other non-descriptive breeds have been reported to possessed higher adaptability, higher resilient to ticks, tick-borne diseases and gastrointestinal nematodes (Ndlovu *et al*., 2007; Muchenje *et al*., 2008).Nguni breeds also have improved feed efficiency and better ability to select improved quality diets from coarse forages on rangelands (Collins-Luswati, 2000). However, years of experience was not a significant predictor of improved practices and thus the link between cattle breed and improved practices might be more complex and possibly a proxy for unmeasured variables in the study.

Farmers that employed a semi-intensive management system had a higher likelihood of implementing positive bovine fasciolosis preventive strategies compared to those farmers

engaging in backyard or extensive systems of management. The farmers engaging in semi-intensive management system might be more likely to seek education and intervention from veterinary personnel. All preventable measures such as avoidance of water-logged pasture, avoidance of early morning grazing, pasture management, rotational grazing and periodical prophylactic treatment and routine deworming with anthelminthic might have been instituted because of a veterinary herd health program. Moutos et al. (2022) similarly reported on the importance of farmers' education for the prevention of zoonotic diseases. Also, veterinary supervision, which might be more likely with semi-intensive systems, has been linked to improved practices for the prevention of zoonotic diseases. Gaps in knowledge and high-risk practices concerning bovine brucellosis have been associated with the absence of veterinary supervision in Portugal (Díez et al. 2013). It is thus important for smallholder farmers to be trained on the epidemiology of bovine fasciolosis to improve their knowledge and practices and thus reduce the negative impact of the disease on their herds.

The study results should be interpreted in conjunction with several limitations because bias in questionnaire studies is inevitable. This is a fundamental issue in public health research and categorized into three ways; challenges associated with question design, whole questionnaire design, and administration of the questionnaire (Choi and Pak, 2005). This issue of bias was minimized by carefully designing each question and pre-testing carried out using farmers in different locations. Correct statements concerning bovine fasciolosis epidemiology and improved practices were mixed with some false statements. Furthermore, bias such as response bias due to self-reporting was beyond our control, especially when the participant wanted to satisfy the researchers by participating in the survey (Rosenman et al. 2011).

3.5 Conclusions

The present study identified that smallholder cattle farmers, especially less educated farmers, and extensive producers, in the North West Province had a poor likelihood of engaging in satisfactory practices of prevention and control of bovine fasciolosis. Training and awareness sessions for smallholder farmers on these aspects are therefore recommended. The findings of this survey might therefore suffer from some social desirability bias. The sample size, non-random selection of participants, and data collection via structured questions might not adequately represent the study population. Language also appeared to be a limitation as interpreters were required, which might not have translated the questions correctly. More so, some farmers were not patient enough to listen attentively before offering their responses. Important data have been collected despite the potential limitations of the current study.

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Chapter 4: Comparison of three diagnostic methods to detect the occurrence of *Fasciola species* in communally grazed cattle in the North West Province, South Africa

(Published in *Pathogens*)

Abstract

Fasciolosis is a parasitic disease of cattle that causes significant economic losses in commercial cattle herds in South Africa but its prevalence is unknown in most communal areas. A cross-sectional study was conducted to determine the occurrence of bovine fasciolosis using three different diagnostic methods in five villages of the Moretele Local Municipality in Bojanala District, North West Province. Faecal samples were collected from 277 cattle of different breeds, ages, sex, and faecal consistency scores and examined using the sedimentation technique, quantitative real-time polymerase chain reaction (qPCR), and faecal antigen enzyme-linked immunosorbent assay (Ag ELISA). All samples were negative for bovine fasciolosis using faecal Ag ELISA. Seventy-three (26.4%) of the 277 samples were positive using the qPCR while 36 (13.0%) were positive using the sedimentation technique, though with low feacal egg counts (1 to 20 eggs per gram). The qPCR detected the highest positivity (26.4%, 95% CI 21.26, 31.96) followed by the sedimentation test. Neither location, breed, sex, age, nor faecal consistency score were associated with positive qPCR results ($p>0.05$). There was also no significant agreement (kappa= -0.011, $p=0.843$) between qPCR and the sedimentation technique for the detection of *Fasciola* spp. qPCR appeared to be the most sensitive method for the detection of *Fasciola* spp. Further studies are required on the characterization of *Fasciola* spp. in communal cattle in South Africa.

Keywords: antigen ELISA, bovine, *Fasciola* species, sedimentation, real-time PCR.

4.1 Introduction

Fasciolosis, also known as distomatosis or liver fluke disease, is an important neglected endo-parasitic zoonosis caused by trematodes of the genus *Fasciola* (Phylum Platyhelminthes: Family Fasciolidae) (Mas-Coma et al., 2005; Mas-Coma et al., 2019). The most common species are *F. hepatica,* common in temperate regions and *F. gigantica,* common in tropical countries (Le et al., 2007; Admassu et al., 2015; Haridwal et al., 2021). Hybrids from both *F. hepatica* and *F. gigantica* have also been reported in some countries including South Africa (Nguyen et al., 2009; Le et al., 2008; Periago et al., 2008; Haridwal et al., 2021). Transmission of *Fasciola* spp. is by freshwater snails of the family Lymnaeidae (Mahulu et al., 2019). In South Africa, *F. hepatica* is mainly transmitted by *Galba truncatula* (De Kock and Wolmarans, 2008), while *F. gigantica* is mainly transmitted by *Radix natalensis* (Moema *et al.,* 2008). *Pseudosuccinea columella* is capable of transmitting both *Fasciola* spp. (Malatji and Mukaratirwa, 2020). Fasciolosis is widely distributed globally and affects humans and a wide range of wild and domestic ruminants (Mas-Coma et al., 2009; Ibrahim, 2017) causing severe losses to livestock production.

Annual productivity losses due to fasciolosis in livestock have been estimated to exceed US$ 302 billion with global annual economic losses exceeding US$ 200 million (Mehmood et al., 2017). Annual financial losses of ZAR 129, 901 (US$ 9,992.40) were estimated due to whole liver condemnation among slaughtered cattle in the Eastern Cape, South Africa (Jaja et al., 2017). Economic losses affect cattle farmers, butchers, and consumers in the form of liver condemnation, reduction in growth rate, poor carcass quality, poor conception rate, and mortality (Mungube et al., 2006; Mehmood et al., 2017). These losses necessitate the evaluation of methods for the detection of the disease and the implementation of mitigation strategies. In South Africa, few

studies have determined the prevalence of fasciolosis and these have been largely abattoir-based (post-mortem diagnosis) and biased toward commercial cattle production. An on-station study by Ndlovu et al. (2009) demonstrated a *Fasciola* spp. prevalence of 16.3 % in cattle on a research farm in the Eastern Cape Province using the formalin-ether sedimentation method. Jaja et al. (2017) reported the highest prevalence of fasciolosis in summer (23 %) and the lowest (5 %) in winter in slaughtered cattle in the same province using post-mortem liver inspection. Recently, Mpisana et al. (2022) reported an overall prevalence of 39.1 % in slaughtered dairy cattle in the Eastern Cape using liver inspection. There is a need for on-farm antemortem detection studies of bovine fasciolosis, especially in communal areas across South Africa where farmers have little knowledge about the disease and cattle might therefore be at a higher risk.

Antemortem detection of *Fasciola* spp. in cattle has traditionally been achieved using sedimentation and faecal egg counts (FEC) techniques or the faecal antigen enzyme-linked immunosorbent assay (Ag ELISA). The sedimentation method detects *Fasciola* spp. eggs in faeces from patent infections, but it is laborious, does not differentiate between the different *Fasciola* spp. and has low sensitivity, and will not detect pre-patent infections (Brockwell et al., 2013). The Ag ELISA can detect *Fasciola* spp. secretory-excretory antigens in faeces even during the pre-patent period but cannot differentiate between the two *Fasciola* spp. and has low sensitivity when FEC are low (Brockwell et al., 2013; Martinez-Sernandez et al., 2016). Quantitative real-time polymerase chain reaction (qPCR) has been developed that can detect *Fasciola* spp. DNA in faecal samples even with very low egg counts (Calvani et al., 2017) and as early as two weeks' post-infection (Martinez-Perez et al. 2012; Robles-Perez et al., 2013). There is a need to compare diagnostic methods for the selection of the most sensitive and specific on-farm method to detect

the occurrence of bovine fasciolosis. Field-based occurrence studies will provide essential antemortem data for the prompt development of fasciolosis control strategies that will mitigate economic losses.

The North West Province has approximately 1,776,000 beef cattle, which is about 12.8 % of the estimated 13,853,000 cattle population of South Africa (Hendriks et al., 2016). Smallholder farmers in the North West Province of South Africa, lack knowledge and have a poor likelihood of executing satisfactory practices on the prevention and control of bovine fasciolosis (unpublished data). Few reported attempts have been made to detect the disease in cattle owned by smallholder farmers in the province. The objectives of the current study were, therefore, to estimate the prevalence of fasciolosis in naturally infected cattle reared communally by smallholder farmers in the North West Province using the sedimentation technique, qPCR and Ag ELISA, and to compare the detection rate across the three methods. It was hypothesised that the qPCR would detect a higher prevalence compared to Ag ELISA or sedimentation.

4.2 Materials and methods

4.2.1 Ethical approval

Ethical clearance was obtained from the Faculty of Veterinary Science Research Ethics Committee (REC086-19), Animal Ethics Committee (REC 086-19), and the Faculty of Humanities Research Ethics Committee (04915365:REC086-19) at the University of Pretoria, South Africa. Permission to conduct research under Section 20 of the Animal Diseases Act (Act 35 of 1984) was provided by the Department of Agriculture, Land Reform, and Rural Development of the Republic of South Africa with reference number 12/11/1/18.

4.2.2 Study area

The study was conducted in Makapanstad, Lekgolo, Tladistad, Mmakaunyane, and GaMotle villages in the Moretele Local Municipality that falls under Bojanala District Municipality in the North West Province of South Africa. The North West is one of the nine provinces of South Africa, located in the north western part of the country (Figure 1). Makapanstad village covers an area of 20.45 km^2 with a total human population of 15,076. Its geographical coordinates are latitude 25° 14' 36" S and longitude 28° 7' 19" E. Ga-Motle is a populated place in Bojanala District Municipality. It is located at an elevation of 1,068 meters above sea level. Its coordinates are 25° 21' 0" S and 28° 4' 0" E. The annual rainfall of these areas is 600 mm received mostly in summer (November – March) and the temperature averages 12° C in the winter season and 25° C in summer (Letsoalo et al. 2000).

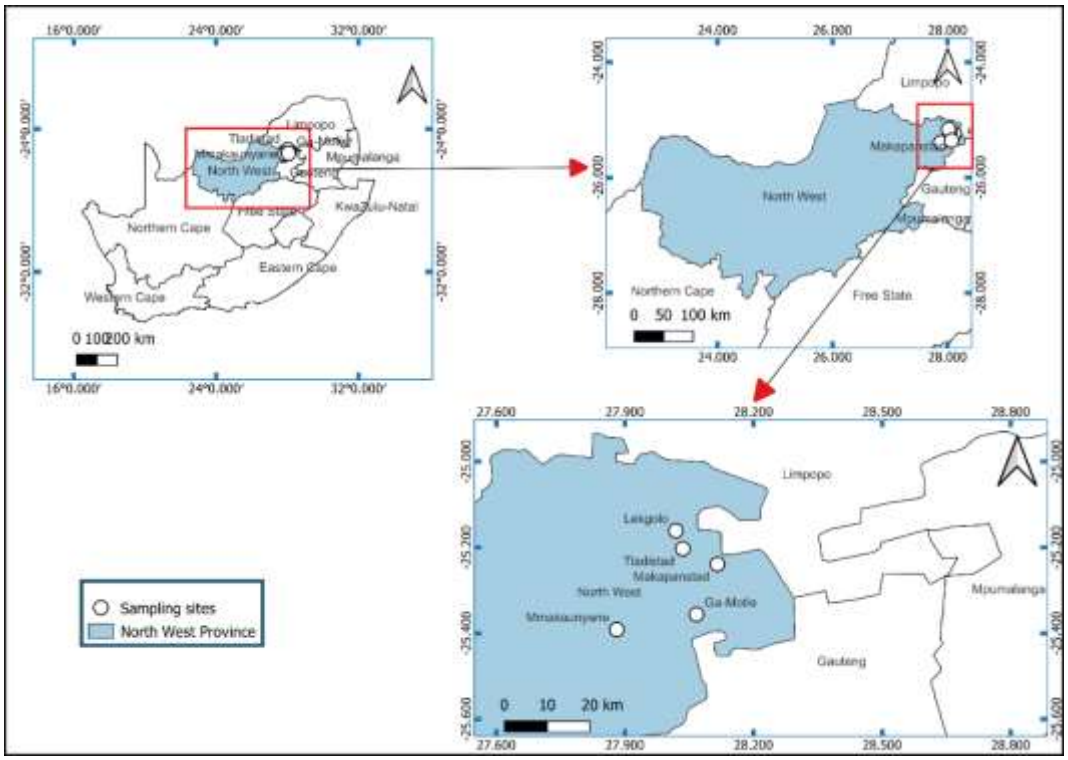

Figure 4.1: Moratele Local Municipality, North West Province showing the study locations marked in white dots.

4.2.3 Study design and sample size

The district and local municipality were selected based on ease of access to cattle herds reared under a communal farming system and the willingness of farmers to participate in the study. Five villages were sampled based on the farmers' commitment to bringing their animals for sampling. Cattle in each herd were selected by random sampling recording the breed, sex, age, and faecal consistency score. Farm records were used to determine age was categorised as 2-4 years (young adults) or above 4 years (adults). Breed type was determined based on phenotypic characteristics (Makina et al., 2016; Sojl et al., 2015) in conjunction with farmer records. Faecal consistency score was graded as poor, average, and good based on Renaud et al. (2020). The sample size was estimated based on the formula by Thrusfield (2018) as follows:

$$n = 1.962\frac{Pexp(1 - Pexp)}{d^2}$$

Where: n= total number of sample size; d= absolute precision (5%); Pexp = expected prevalence. An estimated prevalence of 23.3% was used based on a previous study on the detection of *Fasciola* spp. using post-mortem liver inspection at abattoirs in the Eastern Cape Province (Jaja et al., 2017). The calculated sample size was 275 cattle.

4.2.4 Collection of faecal samples and animal records

Feacal samples were collected using a lubricated gloved hand from the rectum of each animal into separate empty faecal containers, which were subsequently labeled with the village name, date of sampling, breed, sex, age, and faecal score. Collected samples were placed in a cooler box with

ice packs and then transported to the Parasitology laboratory of the Department of Veterinary Tropical Diseases at the University of Pretoria, South Africa for analysis.

4.2.5 Sedimentation technique to identify fluke eggs

Eggs of *Fasciola* spp. were identified and enumerated using a sedimentation technique as described by Calvani et al. (2017) with some modifications. Six grams of faeces were homogenized with 20 ml of distilled water using a wooden spatula. The mixture was filtered using distilled water from a high-pressure source into a 95 µm filter and placed in another 50 µm filter. The residue from the 50 µm filter was collected into a 1000 ml glass beaker by rinsing it with water. The beaker was filled with water and left to stand for 5 minutes. The supernatant was decanted, followed by refilling of the beaker with distilled water, and left to stand for another 5 minutes. After the second rinsing, most of the supernatant was decanted and the remaining mixture was poured into a measuring cylinder (of capacity 100 ml) and left to stand for 5 minutes. The supernatant was again decanted to leave a sediment of about 10 ml and two drops of methylene blue (1%) were added to the sediment and mixed by shaking. Examination for *Fasciola* eggs was performed under a light microscope at 10 x magnification (Olympus microscope, New York microscope company, Hicksville, NY 11801, USA). The number of *Fasciola* spp. eggs in all grids in the counting chamber were counted and recorded. Happich (1969) demonstrated that about one-third of eggs from the initial faecal sample volume are retained in the final processed sediment. Therefore, the value of eggs per gram (EPG) for each faecal sample was calculated by multiplying by 3 the number of eggs counted and then dividing this by 6 (the initial grams of faeces). The 10 ml sediment was collected in a sterile plastic container for subsequent DNA extraction, which was done on the same day.

4.2.6 Molecular analysis

4.2.6.1 DNA extraction

DNA was extracted from the faecal sediments (n=256) using a QIAamp ® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) as described by Calvani et al. (2017), with some modifications. The faecal sediment was poured into a 15 ml plastic tube and centrifuged at 4,000 rpm for 40 minutes. The pellet was collected using a sterile fine wooden applicator stick and placed into a pre-prepared tube containing ceramic beads (MagNA Lyser Green Beads, Roche Diagnostics, Mannheim, Germany) and 700 µl of Lysis buffer, AL (QIAGEN, Hilden, Germany). The mixture was homogenised at 6800 revolutions per second for 36 seconds in a Precellys 24 Tissue Homogeniser (Bertin Technologies SAS, Montigny-le-Bretonneux, France). This was followed by incubation at 85 °C for 10 minutes. About 600 µl of the mixture was transferred into a 2 ml microcentrifuge tube containing 25 µl proteinase K, followed by the addition of 700 µl of InhibEx. The mixture was vortexed for 15 seconds and then incubated at 70 °C for 24 hours. The rest of the DNA extraction procedure, starting with the addition of ethanol, was as described in the QIAamp ® Fast DNA Stool Mini Kit protocol (Calvani *et al*., 2017). Extracted DNA was stored at -20°C until further analysis.

4.2.6.2 Quantitative real-time PCR for *Fasciola* species

A qPCR assay (Alasaad et al., 2011) was used for the detection of *Fasciola* DNA in faecal samples. Oligonucleotide primers SSCPFaF (5'-TTG GTA CTC AGT TGT CAG TGT G-3')
and SSCPFaR (5'-AGC ATC AGA CAC ATG ACC AAG-3') were used to amplify a 140 base pair (bp) fragment of the internal transcriber spacer 2 (ITS-2) gene for *Fasciola* spp., and species specific TaqMan probes ProFh (5'-FAM-ACC AGG CAC GTT CCG TCA CTG TCA CTT T-

QSY-3') and ProFg (5'-VIC-ACC AGG CAC GTT CCG TTA CTG TTA CTT TGT C-QSY-3') were used to amplify *F. hepatica* and *F. gigantica* DNA, respectively. Each PCR reaction comprised 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa), 0.3 µM of each primer, 0.1 µM of the FAM and VIC-labelled probes, and 2 µl of DNA template in a total reaction volume of 20 µl. Thermal cycling was done in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa) under the following conditions: Uracil N-Glycosylase digest at 50 °C for 2 minutes, followed by AmpliTaq Gold pre-activation at 95 °C for 10 minutes and then 45 cycles of amplification at 95 °C for 15 seconds and annealing at 60 °C for 1 minute. Positive controls were DNA previously extracted from adult *Fasciola* worms obtained from infected livers, confirmed using PCR and DNA sequencing. The negative control was nuclease-free water.

4.2.7 Ag ELISA

The *F. hepatica* antigenic indirect Sandwich ELISA kit (Bio-X Diagnostics, Rochefort, Belgium) was performed as described by the manufacturer. Rows A, C, D, and G of the microtiter plate were coated with polyclonal antibody that is specific to *F. hepatica*. The other rows (B, D, F, and H) were coated with polyclonal antibodies that are not specific to the parasite. The faecal material was diluted by adding 2 ml of the dilution buffer to 2 grams of the faecal material, afterwards, the diluted sample was centrifuged at 1,000 g for 10 minutes. To each well, 100 µl of the supernatant of each sample was added. Samples were added as follows: sample 1 in wells A1 and B1, and the other samples and controls were added in that order. The plates were incubated at 24 °C for 2 hours on a rotatory incubator (Environmental Shaker-Incubator ES-20, Biosan Ltd, Germany). Afterward, the plates were washed three times with the wash buffer and 100µl of the diluted biotin-

linked anti-*Fasciola hepatica* conjugate was added to each well. The plates were incubated at 24 °C for 1 hour on the rotatory incubator and were washed three times afterward. Following the wash step, 100 µl of the avidine-peroxidase conjugate was added to each well and incubated at 24 °C for 1 hour. Subsequently, the plates were washed three times and 100 µl of the chromogen was added to each well on the plates. The plates were incubated in the dark at room temperature for 10 minutes. Afterward, 50 µl of stop solution was added to each well. The optical densities (OD) were read at 450 nm immediately after the addition of the stop solution (BioTek Power Wave XS2microplate reader, Agilent Technologies Inc, USA). The net OD of each sample was calculated by subtracting the reading of wells A, C, D, and G from the corresponding negative control (B, D, F, and H). The change in OD for each well was divided by the corresponding positive control and multiplied by 100 to express the result as a percentage of the positive control. Samples with values greater than 8% were considered positive.

4.2.8 Data analysis

Descriptive statistics were performed to determine the distribution of FEC (expressed as eggs per gram) and quantification cycles (C_q) from the sedimentation technique and qPCR, respectively. The qPCR assay was considered the gold standard for the diagnosis of *Fasciola* spp. Apparent prevalence and corresponding 95% confidence intervals were calculated based on the proportion of results positive on each diagnostic assay. Univariate analysis was used to estimate the association between qPCR status for *Fasciola* spp. (positive, negative) and potential risk factors (location, breed, age, sex) using chi-square tests. A generalized linear model was used to determine the association between faecal consistencies - categorized as poor, average, and good (Renaud et al., 2020) with the presence of *Fasciola* spp. DNA (qPCR determined). The Cohen's Kappa test

was used to determine the level of agreement across three methods (sedimentation, qPCR, antigen ELISA). The kappa value was categorized as poor (<0.00), slight (0.00-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) and almost perfect (0.81-1.00) as previously described (Landis and Koch, 1977). Data were analysed using R software version 4.0.5 (Team, 2013) and results were interpreted at the 5 % significance level.

4.3 Results

4.3.1 Descriptive of the sampled cattle

A total of 277 cattle were sampled for faeces in five locations as follows; (Legkolo [n=50], GaMotle [n=60], Makayauna [n=65], Tladistad [n=55], Makapanstad [n=47]). An average of five cattle were sampled per herd and a maximum of 10 herds were sampled per village. Most cattle were non-descript crossbreds (n=143), followed by Brahman (n=90) and Afrikaner (n=32) breeds (Table 1). Other breeds were: Bonsmara (n=4), Boran (n=1), Charelac (n-1), Limosine (n=2), Nguni (n=3), and Simental (n=1). The highest number of sampled cattle (n=153) were 2 to 4 years of age, followed by above 4 years old animals (n=124). There were fewer males sampled (n=31) than females (n=246).

4.3.2 Presence of *Fasciola* eggs in faecal samples using the sedimentation technique

The sedimentation technique detected *Fasciola* spp. eggs in 36 (13.0%) of the 277 cattle faecal samples examined. The FEC per sample was generally low; 30 of the 36 animals (83%) had 1 to 4 eggs per gram (EPG), while three animals each (8%) had 5 to 10 EPG or above 10 EPG respectively.

4.3.3 Occurrence of *Fasciola* species in faecal samples using qPCR

Of the 277 cattle sampled from the five locations, 73 (26.4%, 95% CI 21.26, 31.96) were positive for *Fasciola* spp. using qPCR. More than half of the qPCR positive samples (n=41) showed quantification cycle (C_q) values greater than 30, while 14 samples had C_q values of 26 to 30, and only 18 samples showed C_q values less than 25 (Figure 2). The four variables (location, breed, age, sex) were assessed in univariate analyses; only location ($p<0.001$) and age ($p=0.045$) were significantly associated with the detection of *Fasciola* spp. (Table 1). The multivariable analysis did not identify any significant predictor or effect on infection with *Fasciola* spp.

Table 4.1: Descriptive analysis and univariate associations between potential animal-level risk factors and *Fasciola* spp. status as determined using the quantitative real-time PCR assay.

Variable (category)	Number of positive cattle (%)	95% CI	p-value
Location			
Makanpastad (n=47)	26 (55.3)	40.12, 69.83	<0.001
Legkolo (n=50)	11 (22.0)	11.53, 35.96	
Makayauna (n=65)	11 (16.9)	8.76, 28.27	
GaMotle (n=60)	8 (13.3)	5.94, 24.59	
Tladistad (n=55)	17 (30.9)	19.14, 44.81	
Breed			
Afrikaner (n=32)	3 (9.4)	1.98, 25.02	0.065
Brahman (n=90)	24 (26.7)	17.89, 37.03	
Crossbreed (n=143)	42 (29.4)	22.06, 37.56	
Sex			
Female (n=246)	62 (25.2)	19.90, 31.11	0.221
Male (n=31)	11 (35.5)	19.23, 54.63	
Age			
2 to 4 years (n=153)	33 (21.6)	15.34, 28.94	0.045
>4 years (n=124)	40 (32.3)	24.15, 41.24	

CI: confidence interval

4.3.4 Association between faecal consistency and presence of *Fasciola* DNA in faeces

About three-quarters (n=206) of faecal samples were of average consistency, 41 of good, and 30 of poor consistency. Results of a generalised linear model to determine the association between faecal consistencies and the presence of *Fasciola* spp. DNA showed that there were no statistically significant differences in positivity between samples with different consistency. The percentage of *Fasciola*-positive samples increased from the faecal samples with a good consistency (17.1%) to those with average (27.7%) and poor consistency (30.0%), although this was not statistically significant ($p>0.05$) (Table 2). Faecal samples with poor or average consistency were twice more likely to be positive for *Fasciola* spp. compared to samples with good consistency, although this was not statistically significant ($p>0.05$) (Table 2).

Table 4.2: Comparison of *Fasciola* quantitative real-time PCR status with the consistency of faecal samples collected from cattle in the North West Province, South Africa.

Faecal consistency	No. of positive samples	% of positive samples	Odds ratio	p-value
Good (n=41) (ref)	7	17.1		
Average (n=206)	57	27.7	1.86	0.162
Poor (n=30)	9	30.0	2.08	0.203

4.3.5 Comparison of detection rate of *Fasciola* spp. between sedimentation and qPCR

There was no agreement above chance (kappa= -0.011, p=0.843) in the detection of *Fasciola* spp. between the qPCR and the sedimentation technique (Table 3). Of the 277 samples tested, 23.5% (n=64) were positive with qPCR but negative with sedimentation, while 9.7% (n=27) were positive with sedimentation but negative with qPCR. Only 9 (3.2%) were positive with both sedimentation and qPCR (Table 3).

Table 4.3: Level of agreement for the detection of *Fasciola* spp. between sedimentation and quantitative real-time PCR

		qPCR n (%)		
		Positive	Negative	Total
Sedimentation n (%)	Positive	9 (3.2)	27 (9.7)	36 (13.0)
	Negative	64 (23.1)	177 (63.9)	241 (87.0)
	Total	73(26.4)	204 (73.6)	277 (100)

4.3.6 Detection of *Fasciola* spp. using Ag ELISA

A total of 204 faecal samples (from the overall 277 samples) were tested for *Fasciola* antigen using an antigen ELISA kit. The Elisa test kit excluded *F.gigantica* and it was only *F.hepatica*. All 204 samples tested negative with Ag ELISA.

4.4 Discussion

Three diagnostic tests were used in the present study to detect the occurrence of *Fasciola* spp. in naturally infected cattle belonging to smallholder farmers in five villages in the North West Province of South Africa to establish the most suitable diagnostic method for on-farm testing of the occurrence of bovine fasciolosis. Calvani et al. (2017) previously demonstrated a good

correlation between qPCR diagnostic workflow and sedimentation in detecting experimental *Fasciola* spp. infection in cattle in Laos. It was not known how this relationship would be influenced by the natural infection of cattle under field conditions and how it compared to detection by the Ag ELISA method. The current study is to the authors' best knowledge the first to compare the detection of *Fasciola* spp. by the three tests in naturally infected cattle under field conditions in South Africa.

The observed variation in detection rate by the three diagnostic methods in the present study was most likely related to differences in the sensitivity of the diagnostic tests. It was expected that the qPCR would detect the largest proportion of positive samples compared to Ag ELISA or sedimentation since it has been previously reported to be the most sensitive diagnostic method (Martinez-Perez et al., 2012; Calvani et al., 2018). The failure of Ag ELISA to detect any positive samples in the present study was unexpected given the number of positive samples detected by the sedimentation test, which is considered to be a less sensitive diagnostic method (Mezo et al., 2004). It is important to note that most of the positive samples on sedimentation in the present study had low egg counts (< 10 EPG), which most likely affected the sensitivity of the Ag ELISA as reported by Brockwell et al. (2013) and Martinez-Sernandez et al. (2016). The current findings support the reports by Novobilský et al. (2012), Kajugu et al. (2015), and Calvani et al. (2017) that the sensitivity of Ag ELISA is generally low in natural compared to experimental infections possibly due to the over-dilution of the faecal antigens below the detection limit of the test in natural infections. Furthermore, Gordon et al. (2012) postulated that unlike in natural infection, the large infective dose of 200 metacercariae in experimental infections contributes to the increased sensitivity of Ag ELISA. Note

The qPCR had higher apparent sensitivity with a greater percentage positivity (26.4%) at detecting *Fasciola* spp. from faecal samples. The DNA isolation protocol and the C_q values recorded in this present study were similar to those previously described by Calvani et al. (2017). The increased sensitivity of this protocol could be attributed to the increased starting volume of faeces (6 grams) and concentration of eggs by faecal sedimentation. Loss of free adult faecal DNA during the washing, nevertheless, might be an inherent weakness of the method (Calvani et al., 2018) and could likely lead to reduced sensitivity, especially in pre-patent infections. Though two separate probes for *F. hepatica* and *F. gigantica* were used in either individual probes or duplex qPCR protocols. This could likely be an indication of mixed *Fasciola* spp. infections (including hybrids) within the positive samples or might indicate poor specificity of the qPCR to differentiate between the species in faecal samples. The later assertion is concerning as the qPCR has been reported to be able to differentiate between *F. hepatica* and *F. gigantica* DNA, though this differentiation was made possible through sequences of the ITS-2 rDNA (Alasaad *et al*., 2011). Calvani et al. (2020) developed single nucleotide polymorphism (SNP) assays targeting the ITS1 and 1s RNA genes of *Fasciola* spp. and confirmed the identity of the infecting *Fasciola* spp. using Illumina sequencing to diagnose infections in faecal samples. To accurately diagnose and differentiate between the two *Fasciola* spp. in naturally infected cattle, there is a need to employ further molecular tools such as cloning and sequencing in conjunction with the qPCR.

Faecal sedimentation with fine filtration and FEC was less sensitive than the qPCR in detecting *Fasciola* spp in faecal samples. The large proportion of sedimentation-positive samples have low FEC and this corroborates the findings of Calvani et al. (2018) who also discovered a higher detection rate using qPCR compared to the sedimentation method in sheep though interspecies

variations are not uncommon as observed by Paras et al. (2018). Caution needs to be taken when interpreting results from cattle herds that have generally low egg counts as it has been shown that the sedimentation/filtering process has an inherent unintended effect of losing or overlooking eggs, which might reduce the sensitivity of the test at the lower detection limit (Reigate et al., 2021). False negative FEC has been reported in field studies (Arifin et al., 2016; Mazeri et al., 2016) and these could be indicative of pre-patent infections or irregular shedding patterns of *Fasciola* eggs via the biliary system in cattle (Brockwell et al., 2013). Duplicate sedimentations could be used to improve the sensitivity of sedimentation as reported by Calvani et al. (2018), though these are more time-consuming especially when processing large numbers of field samples. Most studies exploring the detection limit of the sedimentation method have been experimental using samples with spiked *Fasciola* spp. eggs hence more studies to establish the detection limit of the test in natural infections are required.

In the present study, only 3.2% of samples were positive with both qPCR and sedimentation and coupled with a negative kappa value, this denoted no agreement in the detection of *Fasciola* spp. between the two methods. The present finding was in discord with the reports of Calvani et al. (2018) who reported a good correlation between the two methods. Arifin et al. (2016) also reported poor agreement in the detection of *Fasciola* spp. between PCR and loop mediated isothermal amplification (LAMP) with sedimentation, though this was considered to be due to the poorer sensitivity of the molecular tests compared to sedimentation. About a quarter of the samples that were negative with sedimentation tested positive on qPCR most likely due to the detection of free worm DNA in faecal samples even in pre-patent infections. Almost 10% of the samples that were positive on sedimentation tested negative with qPCR possibly due to the reduced specificity of the

sedimentation method. Concurrent *Paramphistomum* spp. infections were observed in cattle in the present study and this might have contributed to the lack of specificity as the eggs have a similar shape to *Fasciola* eggs; however, the trained eye should be able to discriminate between the two kinds of eggs as they are of a different colour (Mazeri et al., 2016).

The inability of the Ag ELISA technique to detect *Fasciola* spp as seen in this present study may be due to the poor sensitivity of Ag ELISA method to detect the presence of *Fasciola* spp during the pre-patent period of parasitism as previously described by (Mezo et al., 2004; Flanagan et al., 2011b; Valero et al., 2009). The generally low FEC observed in samples in the present study might have also affected the sensitivity of the Ag ELISA. Poor agreement between Ag ELISA and qPCR observed in the current study could be related to the failure of the Ag ELISA to detect *Fasciola* spp. during the patent phase especially in natural infections. The present result is in contrast to that of Calvani et al. (2018) who reported a good correlation between Ag ELISA and qPCR. It must be noted that the qPCR protocol in the present study was linked to the sedimentation protocol which most likely made it more efficient in detecting patent infections and possibly reduced its correlation with the Ag ELISA. The Ag ELISA may be suitable for detecting adult liver fluke infections but is unreliable against immature liver fluke infections (Gordon et al., 2017).

The prevalence of bovine fasciolosis observed in the present study was relatively higher than the prevalence of 5%-23% detected by Jaja et al. (2017) and greatly lower than the prevalence of 16.4% to 73.6% detected by Mpisana et al. (2022) in the Eastern Cape Province of South Africa. The differences between studies might be related to differences in location, study types, and diagnostic approaches employed by the two earlier studies compared to the current study. The earlier studies focused on cull dairy cattle coming from an irrigated pasture-based dairy system in

the coastal province of the Eastern Cape. These study animals could have been suffering from a higher fasciolosis burden due to higher pasture contamination than the communally grazed beef cattle from the inland province of the North West Province. Mochankana and Robertson (2018) reported a similar lower fasciolosis prevalence in communal than commercial cattle probably due to a lack of access of communal cattle to infected drinking water. Furthermore, the earlier studies were abattoir-based utilising post-mortem liver examination, which is considered extremely sensitive for liver fluke diagnosis while the present study was farm-based utilizing the qPCR or sedimentation and potentially less sensitive (Mazeri et al., 2016). The 13% prevalence of fasciolosis in the present study as determined by the sedimentation technique was similar to that of a previous on-stataion study by Ndhlovu et al. (2009) who used the ether sedimentation technique for the diagnosis of liver fluke. The sedimentation test, thus, is capable of detecting natural *Fasciola* spp. infections and it should not be discarded in antemortem on-farm investigations.

The significant association between the detection of *Fasciola* species and age with a greater likelihood of *Fasciola* spp. infection in older compared to young grazing cattle is possibly related to increased length of exposure to infection on pasture in older cattle. This agrees with the findings of Opio et al. (2021) and Kouadio et al. (2020) who reported a significantly higher *Fasciola* infection rate in cattle aged 4 to 5-years-old compared to younger cattle. The present findings are inconsistent with the previous observation of Phiri et al. (2005) in Zambia where younger cattle had a higher prevalence of fasciolosis than older cattle. Though post mortem liver inspection and coprological examination were the methods adopted in their study. The reason for this may be most likely due to older cattle developing acquired immunity that resulted in resistance to the

flukes. This may also be because younger cattle might shed more eggs while older cattle are more likely to have worms detected in the liver.

The greater likelihood of Brahman and crossbred cattle to be infected with *Fasciola* spp. than Afrikaner cattle observed in this study with qPCR technique may be due to higher innate resistance of the indigenous Afrikaner breed. This also agrees with the report of Mochankana and Robertson (2018) in their cross-sectional prevalence study of *F. gigantica* infections in beef cattle in Botswana where indigenous Nguni cattle were less susceptible to infection than Brahman cattle and their crosses.

Some limitations encountered during this study included, lack of a representative sample from the entire province, relatively small sample size, inability to perform duplicate testing, and sometimes inability to morphologically differentiate between Fasciola and Paramphistomum eggs. The coprological Ag ELISA used was developed for the detection of *Fasciola hepatica* and might not accurately detect *F. gigantica* infection.

4.5 Conclusions

The qPCR was the most sensitive diagnostic test for on-farm detection of bovine fasciolosis followed by sedimentation while the Ag ELISA failed to detect *Fasciola* spp. in the current study. There was a relatively moderate (26.4%) prevalence of bovine fasciolosis in communally grazed cattle in the North West Province as determined by qPCR. Further studies are required for the characterization of *Fasciola* spp. using sequencing techniques in communal cattle in South Africa.

4.6 References

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Chapter 5: The effect of *Fasciola* infection on the hematological and biochemical parameters of cattle reared by smallholder farmers in the North West Province, South Africa

Abstract

The magnitude of production losses associated with bovine fasciolosis in communal farming areas is unknown. The current study compared the hematological and biochemical profiles of *Fasciola*-shedding and non-shedding cattle (through fecal eggs count) reared by smallholder farmers in the North West Province of South Africa. Faecal, blood, and serum samples were collected from 275 cattle in Moretele Local Municipality communities of North West Province for the detection of *Fasciola* species infection using the sedimentation technique. Haematological and serum biochemical parameters were determined using an Auto hematology analyzer (ADVIA® 120 Hematology system). The mean corpuscular haemoglobin concentration was significantly higher ($p = 0.016$) in shedding cattle compared to the non-shedding group, but no other significant differences were identified in other parameters. There were no significant differences in all the biochemical parameters between shedding and non-shedding cattle. There is a significant association between EPG counts in shedding cattle and levels of Mean corpuscular volume and mean corpuscular haemoglobin concentration with Spearman's rho values of (-0.400) and (0.417) respectively. The lower and upper limit of their 95% CI was calculated as -0.679 and -0.020, and -0.041 and 0.690 respectively. Bovine fasciolosis was associated with a a relative decrease in the values of most of the indices but did not significantly alter the erythrogram, leucogram, and serum chemistry of communal cattle.

Keywords: bovine fasciolosis, communal farmers, gamma glutaryl transferase, haematocrit, haemoglobinaemia

5.1 Introduction

Two trematode (liver fluke) species, *Fasciola gigantica* and *Fasciola hepatica*, and their hybrids or intermediate stages cause bovine fasciolosis, a systemic illness in cattle (Haridwal et al., 2021). It is a new and re-emerging zoonosis with significant concerns for food safety (Oyindamola et al., 2017). Due to decreased productivity and condemnation of animal viscera as a result of the illness, it is associated with huge financial losses (Mehmood et al., 2017). Jaja et al. (2017) earlier reported financial losses attributed to bovine fasciolosis in slaughtered cattle in South Africa to be within the range of ZAR 1,940.8 (2,357 USD) to ZAR 129,901 (9992.4 USD) per annum. Early diagnosis of fasciolosis based on the recognition of clinical signs is important in identifying the disease as a cause of production losses and ensuring early treatment of livestock (Williams, 2020).

Fasciolosis in cattle occurs in two phases, the parenchymal phase in which immature larvae migrate through the liver parenchyma, and the biliary phase in which adult flukes reside in the bile ducts (Behm and Sangster, 1999). The former is associated clinically with acute fasciolosis, especially in calves that ingest large numbers of metacercariae on pasture and is characterised by acute anaemia, hypoalbuminaemia, and death caused by larval migration in the liver (Mitchell, 2002). This migration results in hepatocellular damage and leakage of liver cytosolic enzymes, especially proteases, into the blood stream and reduced synthesis of liver proteins (Williams, 2020). The most prevalent clinical form of the condition in cattle is chronic fasciolosis, which is caused by adult liver flukes in the bile ducts and is characterized by weight loss, anaemia, eosinophilia, and hypoalbuminaemia (Mitchell, 2002). Clinical pathological changes in the

haematological and biochemical parameters of cattle may differ in each phase of the disease and could be used as diagnostic and prognostic indicators in infected cattle.

An increase in liver cytosolic enzymes (such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glutamate dehydrogenase (GDH)) in the blood during the parenchymal phase of acute *Fasciola* infection is brought on by hepatic injury (Nasreldin and Zaki, 2020). On the contrary, chronic fasciolosis causes an elevation of enzymes linked with biliary obstruction (such as gamma-glutaryl transferase) and reduced albumin (a protein made solely by the liver) in the blood (Nasreldin and Zaki, 2020; Brahmbhatt et al., 2021). Reductions in hemoglobin (Hb), total erythrocyte count, packed cell volume, lymphocytes, and monocytes, as well as an increase in total leucocyte count (TLC), mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, neutrophils, and eosinophils, are among the hematological changes linked to bovine fasciolosis (Brahmbhatt et al., 2021). The detection of liver fluke eggs in faeces is indicative of patent infections i.e., adult flukes laying eggs in bile ducts, signaling that the liver has already been subjected to pathological damage.

Therefore, it is important to compare these parameters, between shedding and non-shedding animals to identify good prognostic indicators of clinical cases and early detection of fasciolosis.

The mechanical and toxic effects of the *Fasciola* organisms on the vascular and biliary systems of the liver are the primary pathogenic symptoms of bovine fasciolosis. Hauptman et al. (2001) found that these two systems are crucial for optimal liver function. Any derangement of the vascular and biliary system will ultimately have effects on liver enzymatic activities, proteins, and bilirubin production. One should not underestimate the value of clinical manifestations, haematological, and biochemical indicators as diagnostic, prognostic, and therapy evaluation tools for naturally occurring bovine fasciolosis. In communal farming areas, cattle farmers might not have heard of the disease or possess little or no knowledge of its clinical manifestation. This study therefore, sought to establish and compare haematological and biochemical parameters of naturally *Fasciola* shedding and non-shedding cattle and also to establish a correlation between faecal egg counts and

other parameters. It was hypothesised that infection with *Fasciola* spp will significantly and negatively alter the haematological and biochemical parameters of cattle under smallholder farming management in the study area.

5.2 Materials and Methods

5.2.1 Ethical approval

Ethical clearance was obtained from the University of Pretoria's Faculty of Veterinary Science Research Ethics Committee (Reference number: REC086-19) and Animal Ethics Committee (Reference Number: REC 086-19). A permit issued under Section 20 of the Animal Diseases Act (Act 35 of 1984) allowing the movement of samples from North West Province to the Department of Veterinary Tropical Diseases Laboratory in Onderstepoort was issued by the Department of Agriculture, Land Reform and Rural Development of the Republic of South Africa (Reference number: 12/11/1/18).

5.2.2 Study area

The study area covered five villages which include; Mmakaunyane, Lekgolo, Kroimkail, Nrokie, and GaMotle villages in the North West Province of South Africa, Moretele Local Municipality, which is a part of Bojanala District Municipality. The North West is one of the nine provinces of South Africa, located in the north western part of the country (Figure 1).

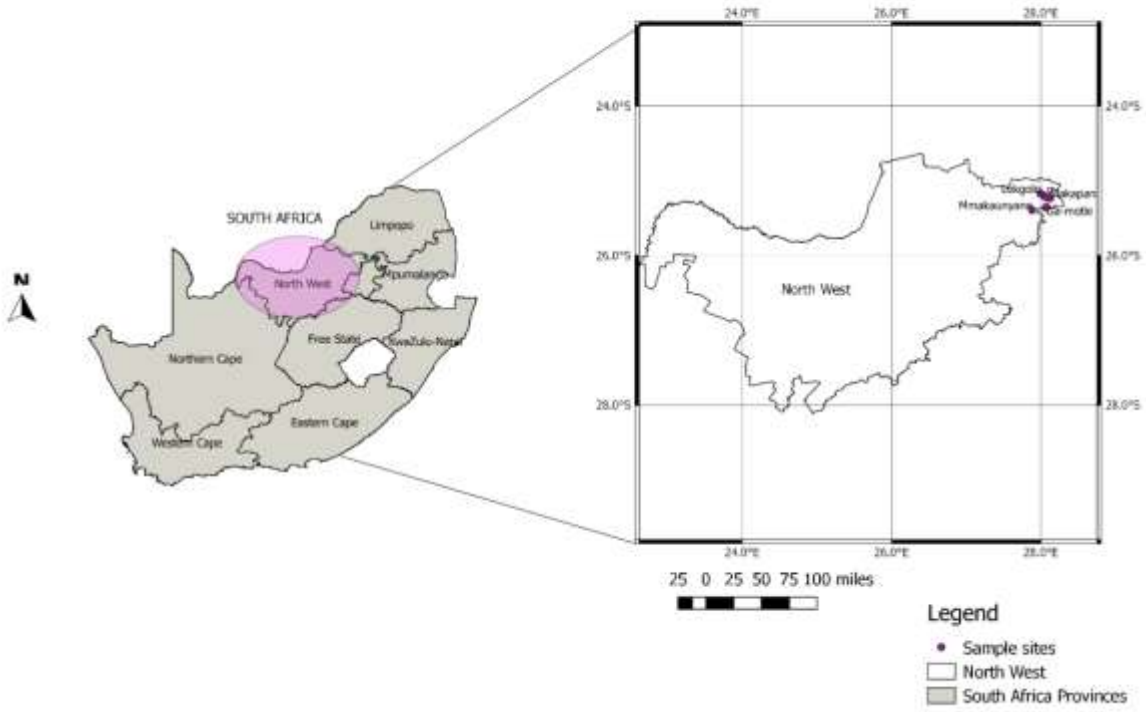

Figure 5.1: A map of the study region, the Moratelle municipality area in South Africa's North West Province. A map of South Africa with the location of the North West Province is included. The pink dots in the Moratelle municipality area represent the sample locations for this study. Map constructed using QGIS software version 2.8

5.2.3 Study animals

The systematic random sampling procedure adopted for sampling. The animals were of different breeds, sex, and age.

The sample size was estimated based on the formula by Thrusfield (2018) as follows:

$$n = 1.962\frac{Pexp(1 - Pexp)}{d^2}$$

Where: n= sample size; d= absolute precision (5%); Pexp = expected prevalence. An estimated prevalence of 23.3% was used based on a previous study on the detection of *Fasciola* spp. using post-mortem liver inspection at abattoirs in the Eastern Cape Province (Jaja et al., 2017). The calculated sample size was 275 cattle.

5.2.4 Sample collection and processing

A total of 275 smallholder cattle had their fresh faeces sampled and taken directly from the rectum into a 50 ml specimen collecting vials with the proper labels. As soon as possible, samples were placed in a cooler box and brought to the lab on the same day of collection. Specimens were held at 4°C at the Parasitology Laboratory in the Department of Veterinary Tropical Animal Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa until examination.

Coccygeal venepuncture was used to collect approximately 15ml of blood from each animal, with 5ml put into sample bottles containing ethylene diamine tetra-acetic acid (EDTA) for hematological analysis. To determine the biochemical indices, 10 ml of the whole blood sample was transferred to a different container without an anticoagulant. These samples were simultaneously collected as the fresh feacal samples were being collected and labeled accordingly. During collection, the samples were kept chilled with ice packs, and they were quickly transported for additional analysis to the Clinical Pathology Unit of the Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria, South Africa. Analysis of the blood was started right away. Without an anticoagulant, samples were centrifuged at 2,500 rpm, the serum was collected, and it was kept at -20 °C until it was needed for biochemistry testing.

5.2.5 Fluke egg sedimentation

Procedures described by Calvani et al. (2017) with some modifications were adopted for the identification and enumeration of eggs of *Fasciola* spp. Six grams of faeces from each animal were measured and homogenized with 20 ml of distilled water using a wooden spatula. The mixture was filtered using distilled water from a high-pressure source into a 95 µm filter placed in another 50 µm filter. The residue from the 50 µm filter was collected into a 1000 ml glass beaker by rinsing with water. After adding water, the beaker was set aside for five minutes. After decanting the supernatant, the beaker was filled with distilled water and let to stand for an additional five minutes. Majority of the supernatant was decanted after the second rinse, and the remaining mixture was then added to a measuring cylinder with a capacity of 100 ml. This mixture was then allowed to stand for 5 minutes. Two drops of methylene blue (1%) were added to the sediment and stirred by shaking after the supernatant was once again decanted to leave a sediment of roughly 10 ml. Using a light microscope (Olympus microscope, New York microscope company, Hicksville, NY 11801, USA), x10 magnification was used to view Fasciola's eggs in the counting chambers for the number of *Fasciola* spp eggs. Happich and Boray (1969) demonstrated that about one-third of eggs from the initial faecal sample volume are retained in the final processed sediment. Therefore, the value of eggs per gram (EPG) for each faecal sample was calculated by multiplying by 3 the number of eggs counted and then dividing this by 6 (the initial grams of faeces). The methylene blue that was added to the sample stained the background material while *Fasciola* eggs appeared golden-yellow.

5.2.6 Haematological analysis

Within six hours of collection of the blood sample, hematologic assays were conducted. Using the ADVIA 120 automated haematology analyzer (Siemens Healthcare Diagnostics, United

Kingdom), parameters such as hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), red blood cells (RBC), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), and platelets (PLT) were determined (Katsogiannou *et al*., 2020). An experienced and board certified clinical pathologist did a 200-cell manual leukocyte differential count. Some of the samples were already haemolysed before processing, and about 209 samples were finally processed.

5.2.7 Biochemical analyses

Using a conventional process and an automatic analyzer, the sera were examined for biochemical parameters such as total serum protein (TSP), albumin (ALB), globulin (GLOB), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), and total bilirubin (TBIL), Cobas-Integra-800-Analyser, Roche Diagnostics, Basel (Roche Company, Switzerland) as previously adopted by Langenmayer *et al*. (2015).

5.2.8 Statistical analyses

SPSS software version 25 was used to analyze the data (Verma, 2012). The biochemical, erythrogram, and leucogram parameters between the Fasciola-shedding and non-shedding samples were compared using the student t-test. P values less than 0.05 were regarded as significant. To ascertain correlations between eggs per gram (EPG) in Fasciola shedding animals, a non-parametric test was performed and haematological and biochemical values were tested by determining the Spearman's correlation coefficient (Spearman's rho) values, level of significance, and the lower and upper limits of their 95 % CI.

5.3 Results

Out of 275 cattle sampled. 66 of the samples got haemolysed before processing and only 209 samples were finally processed, analysed, and computed. Location distribution indicates as follows; Kroimkail 42 (20.1%), Legkolo 32 (15.3%), Makayauna 47 (22.5%), GaMotle 43(20.6%), and Nrokie 45 (21.5%). Breed distribution reveals as follows; Afrikaner 25 (12%), Bonsmara 3 (1.4%), Brahman 66 (31.6%), Cross/Non-descriptive 112(53.6%), and Nguni 3 (1.4%). Sex distribution reveals as follows; Male 187 (89.5%), and Female 22 (10.5%). The age distribution shows the following; less than 2½ years (calf) 11 (5.3%), 2½ to 5 year 145 (69.4%), above 5 to 7½ years 45 (21.5%), and above 7½ years 8 (3.8%) (Tables 5.1, 5.2, 5.3 and 5.4).

5.3.1 Erythrocytic, Leucocytic and Biochemical profiles

The MCHC of the shedding and non-shedding cattle differed significantly (p=0.016). The leucocytic parameters of shedding and non-shedding animals did not differ significantly. All of the biochemical measures between the shedding and non-shedding group showed no significant difference and all parameters were within normal reference ranges (Tables 5.5, 5.6, and 5.7).

5.3.2 The correlation between eggs per gram of *Fasciola* shedding cattle and haematological and biochemical indices

Analysis of the correlation of all animals including Fasciola shedding and non-shedding cattle with every tested parameter reveals no significant correlations. Whereas in the *Fasciola* shedding cattle, MCV and MCHC correlated with higher feacal egg counts, no correlation was recorded for the other parameters. The spearman's correlation coefficient reveals MCV and MCHC with Spearman's rho values of (-0.400) and (0.417) respectively. This indicates a weak negative correlation between EPG counts and MCV parameters which implies that, the higher the EPG counts in Fasciola shedding cattle, the lower their MCV values. The Spearman's rho value of 0.417

represents a relatively weak positive correlation between EPG counts and MCHC values and this implies that as the egg per gram counts in Fasciola shedding cattle increases, there is a mild increase in MCHC values. The lower and upper limit of their 95% CI was calculated as -0.679 and -0.020, and -0.041 and 0.690 respectively. Statistical significance was only observed in MCV and MCHC when correlation analysis was carried out for all parameters tested with P values of 0.035 and 0.027 respectively (Table 5.8).

Table 5.1: Location and distribution of 209 *Fasciola* shedding and non-shedding cattle in communal areas of North west Province South Africa during April- Aug, 2020.

Location	Frequency	Percentage (%)
Kroimkail	42	20.1%
Legkolo	32	15.3%
Makayauna	47	22.5%
GaMotle	43	20.6%
Nrokie	45	21.5%
Total	209	100.0%

Table 5.2: Breeds distribution of 209 *Fasciola* shedding and non-shedding cattle in communal areas of North west Province South Africa during April- Aug, 2020.

Breed	Frequency	Percentage (%)
Afrikaner	25	12.0%
Bonsmara	3	1.4%
Brahman	66	31.6%
Cross	112	53.6%
Nguni	3	1.4%
Total	209	100.0%

Table 5.3: Sex distribution and frequency of occurrence of 209 *Fasciola* shedding and non-shedding cattle in communal areas of North west Province South Africa during April- Aug, 2020.

Sex	Frequency	Percentage (%)
Male	187	89.5%
Female	22	10.5%
Total	209	100.0%

Table 5.4: Age group distribution and frequency of occurrence of 209 *Fasciola* shedding and non-shedding cattle in communal areas of North west Province South Africa during April- Aug, 2020.

Age (in years)	Distribution/frequency	Percentage
Less than 2½ (calf)	11	5.3%
2½ to 5years (young)	145	69.4%
Above5 to 7½ (Adult)	45	21.5%
Above7½ (Old)	8	3.8%
Total	209	100%

Table 5.5: Mean±SD of the Erythrogram of the 209 *Fasciola* shedding and non-shedding cattle in communal areas of North west Province South Africa during April- Aug, 2020 (Mean±SD).

Erythrocytic Parameter	Shedding group n=28	Range	Non-shedding group n=181	Range	P-values
Hb count(g/l)	108.07±14.41	78.00-139.00	108.20±12.87	69.00-143.00	0.998
RBC count(x10^{12}/L)	7.11±1.38	4.83-11.14	6.96±1.04	3.94-11.17	0.266
PCV (L/L)	0.30±0.03	0.23-0.37	0.31±0.08	0.19-1.27	0.669
MCV(fl)	42.90±4.99	27.80-52.80	43.44±5.01	17.70-56.10	0.857
MCH(pg)	15.46±1.83	10.50-19.10	15.72±1.74	11.50-21.10	0.883
MCHC (g/dl)*	36.13±1.02	33.40-38.10	36.10±1.44	31.70-38.50	0.016
RBC distribution width %	19.00±18.97	16.40-23.20	18.49±1.98	1.91-24.20	0.884

Statistical significance between the shedding group and non-shedding group; *p<0.05

MCHC is significantly higher in shedding cattle compared to non-shedding cattle. There are no significant difference (p>0.05) between the other erythrogram parameters of the negative and positive groups.

Table 5.6: Mean±SD of the Total and Differential Leucocytic Counts of the 209 *Fasciola* shedding and non-shedding cattle in communal areas of Northwest Province South Africa during April- Aug 2020 (Mean±SD).

Leucocytic Parameter	Shedding group n=28	Range	Non-shedding group n=181	Range	P-values
WBC (x10^{9}/L)	12.09±4.49	5.45-24.51	12.12±3.64	28.12-12.11	0.067
Segmented Neutrophil (x10^{9}/L)	2.61±1.41	0.76-6.37	4.20±20.28	0.15-275.00	0.266

Band neutrophil ($x10^9$/L)	0.00±0.00	0.00-0.00	0.01±0.06	0.00-0.72	0.080
Lymphocyte ($x10^9$/L)	7.29±2.95	3.00-14.60	7.49±2.63	1.62-21.37	0.148
Monocyte ($x10^9$/L)	0.85±0.39	0.22-1.72	0.84±0.37	0.10-2.96	0.786
Eosinophil ($x10^9$/L)	1.28±1.02	0.27-5.64	1.14±0.76	0.00-3.66	0.592
Basophil ($x10^9$/L)	0.05±0.09	0.00-0.39	0.04±0.08	0.00-0.45	0.238
Other (%)	0.0004±0.002	0.00-0.01	0.001±0.01	0.00-0.18	0.450
Platelet ($x10^9$/L)	162.21±101.19	31.00-322.00	152.80±104.86	11.00-454.00	0.825

Table 5.7: Mean±SD of the Biochemical parameters of the 209 *Fasciola* shedding and non-shedding cattle in communal areas of Northwest Province South Africa during April- Aug 2020 (Mean±SD).

Biochemical Parameter	**Shedding group n=28**	**Range**	**Non-shedding group n=181**	**Range**	**P-values**
Total serum protein (g/L)	72.04±5.78	57.70-80.70	74.03±5.58	60.00-88.80	0.796
Albumin(g/L)	32.60±3.53	24.80-37.90	33.00±3.23	20.40-41.80	0.685
Globulin(g/L)	39.43±6.72	27.10-50.60	41.04±6.41	26.70-55.50	0.816
Gamma-glutamyl transferase(U/L)	15.64±8.23	0.00-35.00	14.72±7.58	0.00-51.00	0.993
Glutamate dehydrogenase(U/L)	12.79±8.20	4.00-41.00	14.59±14.44	0.00-149.00	0.546

Total bilirubin(umol/L)	1.27±0.69	0.20-3.30	1.45±0.72	0.10-3.90	0.314

Table 5.8: Association of eggs per gram of Fasciola shedding cattle and erythocytic parameters in communal areas of Northwest Province South Africa during April- Aug 2020 (Mean±SD).

Erythrocytic Parameter	**Spearman's rho**	**Significance(2-tailed)**	**95% Confidence Intervals (2-tailed) [a,b] Lower**	**Upper**
Hb count(g/l)	.065	.742	-.326	.437
RBC count(x10^{12}/L)	.265	.173	-.131	.588
PCV (L/L)	.015	.941	-.371	.395
MCV (fl) *	-.400	.035	-.679	-.020
MCH (pg)	-.291	.132	-.607	.103
MCHC (g/dl) *	.417	.027	.041	.690
RBC distribution width %	.019	.925	-.367	.399

[a] Estimation is based on Fisher's r-to-z transformation. [b] Estimation of standard error is based on the formula proposed by Fieller, Hartley, and Pearson. [c] Cannot be computed because at least one of the variables is constant.

5.4 Discussion

Having a proper understanding of the haematological and biochemical parameters of fasciolosis-infected cattle is of utmost importance in the treatment decision and control of infected cattle, especially in South African communal farmers' herds of cattle. When compared to the non-shedding group, the values of HB, PCV, MCV, and MCH in *Fasciola* shedding animals were relatively lower, which is suggestive of normocytic anemia. While the results for Hb were similar to those observed in cattle by Molina et al. (2006), this finding is consistent with anaemia in *Fasciola*-infected cattle as reported by Teleb et al. (2007). The decreases in these parameters may

be related to the migration of immature liver flukes, which may cause hepatic blood vessels to burst and subsequent hemorrhages (Urquhart et al., 1996). Reduced PCV and Hb levels in infected cattle may also result from persistent liver inflammation, which suppresses erythrogenesis (Lotfy et al., 2003). Egbu *et al*. (2013) have previously linked anaemia with *Fasciola* worm burden.

The relatively increased values of RBC distribution width and significantly higher MCHC in infected cattle in the present study are inconsistent with previous reports by Egbu *et al*. (2013) who observed that infected cattle had considerably higher MCV and MCH values than uninfected cattle. El-Aziem Hashem and Mohamed (2017) also noted that there were no appreciable variations in the erythrocytic indices (MCV, MCH, and MCHC) of cattle in the presence or absence of *Fasciola* infection suggesting resilience of cattle to infection. The generally lower values of WBC, segmented neutrophils, band neutrophils, and lymphocytes observed in the *Fasciola*-shedding group in the current study are similar to earlier findings of Ikenna-Ezeh et al. (2019), which showed that infected animals had significantly lower white blood cell counts in comparison to uninfected cattle. The present finding however conflicts with that of Egbu et al. (2013), who found that Fasciola-infected mice had greater leucocyte counts than animals that were not infected. Other concurrent diseases affecting the *Fasciola*-shedding cattle might have reduced their leucocyte counts. The findings might also be due to the chronicity of the infection as low leucocyte counts have been reported to be associated with the chronic infective stage of infection. This may also be due to the subclinical nature of fasciolosis as this study was from herds that were apparently healthy. There have been previous reports of cattle having reduced chances of manifesting clinical signs of fasciolosis compared to small ruminants as a higher infection risk of metacercariae is required for clinical disease manifestation (Dargie, 1987; Mitchell, 2022). This subclinical nature

has been linked to a relatively bigger size of cattle liver, with a more fibrous texture and greater functional reserve potential (Mitchell, 2002).

Although there were no significant increase in the values of monocytes, eosinophils, basophils, and platelets, their values were relatively higher in the *Fasciola*-shedding group. This could be the body's response to the obstructive effects of *Fasciola* or a result of bone marrow lesions brought on by toxins (Penny et al. 1996). Generalized leucocytosis, a symptom of an immunological response to parasite antigens, is widespread in parasitic illnesses (Coles, 1980).

Fasciola-shedding cattle generally had lower biochemical indices compared to non-shedding cattle and this was characterised by hypoproteinaemia, hypoalbuminaemia, hypoglobunaemia, reduced glutamate dehydrogenase, and hyperbilirubinemia. This might be due to the deleterious effects of fasciolosis on the liver of infected cattle. However, higher values for gamma-glutamyl transferase activity were observed for the *Fasciola*-shedding cattle, though they continued to be within the reference values. Teleb et al. (2007) showed that levels of liver enzymes, total bilirubin, gamma globulins, and creatinine were increased in the serum of *Fasciola hepatica*-infected sheep. Matanovic *et al*. (2007) also reported similar results in cattle infected with *Fasciola gigantica*.

The observed weak negative correlation in the values of MCV with EPG in *Fasciola* shedding cattle may probably be a result of chronic liver inflammation associated with increased worm load which incites depression in the process of erythropoiesis (Lotfy *et al*. 2003). A similar negative correlation between increased EPG and erythrocytic parameters was reported by Egbu *et al*. (2013). The weak positive correlation between EPG counts and MCHC values in the *Fasciola*-shedding group is in tandem with the observation of Brahmbhatt et al. (2021) who reported a corresponding increase in the values of MCHC as the EPG of *Fasciola*-infected cattle increases.

This may be associated with erythrocytes' compensatory mechanism to increase their capacity to carry oxygen, especially during chronic infection. The present finding is however, contrary to the observation of Egbu et al. (2013) who observed no significant correlation between EPG counts and MCHC values in *Fasciola*-infected sheep and non-infected sheep. This difference may be associated with likely species variation or due to variation in analysis. The present finding is based on the *Fasciola* shedding group alone not the infected and non-infected in the previous study.

No significant relationship was observed between EPG counts, except in the values of MCV and MCHC. The neutrophilia, eosinophilia, and generalized leucocytosis were also observed to be proportional to increased EPG counts. This may be due to inflammation and infection resulting from the activity of the flukes in the bile ducts as mentioned by Radostits *et al*. (2000). Moreover, eosinophilia has been reported to be proportional to the degree of antigenic stimulation or parasitic load in helminth infections in general (Ackerman *et al*. 1981).

5.5 Conclusions

The value of MCHC was significantly higher in the *Fasciola*-shedding group compared to the non-shedding group. All other parameters were within the normal reference values, but with decreased values of Hb, PCV, MCV, MCH, WBC, segmented neutrophils, and lymphocytes in *Fasciola*-shedding group compared to non-shedding cattle. It can be concluded that Fasciola infection did not significantly alter the haematochemical parameters of the cattle most likely due to its subclinical nature as observed in the present study.

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Chapter 6: Sequence and phylogenetic analysis of *Fasciola* species from faecal samples of cattle from a communal farming area in the North West province, South Africa

Abstract

Fasciolosis is a zoonotic illness with significant economic implications worldwide. This work used sequence analysis of the mitochondrial cytochrome oxidase 1 (CO1) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) genes to identify *Fasciola* species in communally grazed cattle in the North West province of South Africa. Out of 277 feacal samples collected, 73 DNA samples were positive for *Fasciola* species using a quantitative real-time PCR assay. These samples were then subjected to ITS-2 conventional and CO1 PCR assays that were specific to *F. hepatica* and *F.gigantica*. This was followed by cloning, sequencing, and maximum likelihood phylogenetic analysis. The ITS-2 PCR identified 30 (41%) positive samples for *Fasciola hepatica*, 25 (34%) for *Fasciola gigantica*, and 18 (25%) positive for both species. Only 12 samples (16%) were positive using the *Fasciola*-specific COI PCR. *F. hepatica* and *F. gigantica* ITS-2 and COI sequences were identical within species but varied across species by 3 to 4 nucleotides. The *F. hepatica* CO1 sequences differed from *F. gigantica* sequences at 25 to 29 base positions. *Fasciola hepatica* ITS-2 sequences are grouped into two different clusters, one cluster with reference sequences from China, Libya, and Peru and the other cluster with a sequence from Spain. The *F. gigantica ITS-2* sequences are grouped into one cluster together with sequences from sheep from Libya and cattle from Chad. The *F. gigantica* CO1 sequences grouped with *F. gigantica* isolate sequences from Zimbabwe, while *F. hepatica* CO1 sequences grouped with *F. hepatica* sequences from sheep and cattle from Japan, Tunisia, Austria, and Ecuador. The detection of both *F. hepatica*

and *F. gigantica* as mixed infections in the cattle might lead to hybridization of *Fasciola* species in the study area in the North West province, South Africa.

Keywords: cattle, CO1, *Fasciola*, *ITS-2*, South Africa

6.1 Introduction

Fasciolosis is an economically significant re-emerging food- and water-borne parasitic zoonotic disease (Rahman et al., 2020; Vázquez et al., 2016). In both animals and people, the disease is caused by *Fasciola hepatica* (, *Fasciola gigantica* Amer et al., 2016), or a hybrid of both species (Le et al., 2008). Because climate and the presence of freshwater Lymnaeid snail species affect the distribution and prevalence of Fasciola species, *F. hepatica* is more prevalent in temperate regions of the world, particularly in Europe, the Americas, and Australia, whereas *F. gigantica* is more prevalent in tropical continents, particularly in Africa and Asia. (Spithill and Dalton, 1998). The illness is acknowledged as a significant barrier to animal production in developing nations, notably those in Africa (Mekroud et al., 2006). Socioeconomic losses include the decreased output of meat and milk, stunted growth, carcass condemnation, high cost for anthelminthic treatment, and livestock or human fatalities (Mas-Coma et al., 2004; Mas-Coma et al., 2009). Over 300 million cattle and 250 million sheep were predicted to have contracted *Fasciola hepatica* worldwide (Nguyen et al., 2012). Worldwide economic losses from both species were expected to be more than US$ 3.2 billion annually, with continents like Africa and Asia suffering the most (Charlier *et al*., 2007). Jaja *et al*. (2017)estimated financial losses of about ZAR 44, 930 (3456.2 USD) per annum only, due to liver condemnation amongst cattle in South Africa.

Globally, fasciolosis in humans has been reported, and it is estimated that more than 180 million people are at risk and that 2.4 million people have contracted the disease in more than 60 different

nations (Mas-Coma et al., 1999). Despite the economic and zoonotic significance of the disease, one of the fascilosis-endemic countries in Africa is South Africa. However, there are few reports on the epidemiology of the disease in the nation. The Eastern Cape Province observed a seasonal frequency of 5 to 23% among cattle utilizing coprological examination and post-mortem liver inspection. (Jaja *et al*., 2017). Another study found that Nguni, Bonsmara, and Angus steers reared on sweet veld in the Eastern Cape using a modified McMaster method had a Fasciola prevalence of 16.3%. (Ndlovu et al., 2009). Only two occurrences of fasciolosis in humans have been identified, two in the Western Cape Province (Black *et al*., 2013) and three in Gauteng Province (Scott and Irving, 1964), probably due to underreporting or misdiagnosis. In the North West Province, where a greater percentage of cattle are owned by communal farmers and are the main source of livelihood, there are anecdotally deleterious effects of fasciolosis with regard to productivity. However, there have been no published reports about fasciolosis amongst cattle in the province. A proper understanding of the species' identities and relative occurrence in the province is important for the preventionof the disease.

Molecular analyses are important tools for the identification of *Fasciola* spp., especially when dealing with unknown intermediate forms with distinct phenotypes and genotypes (Hayashi *et al*., 2015). Both *Fasciola* species are aspermic and have mixed sequences, according to research on the nucleus phosphoenolpyruvate carboxykinase and DNA polymerase delta genes conducted in South, Korea, and Japan (Itagaki et al., 2005). This indicates that the flukes are offspring of the two species' hybridization (Shoriki *et al*., 2016). Additionally, it has been noted that both species have hybridized in subtropical areas, resulting in the creation of intermediate forms with mixed phenotypic traits and genetic structure (Le et al., 2008; Itagaki et al., 2011). In South Africa, reports

of *F. gigantica* and *F. hepatica* have been published (Malatji et al., 2020 and have shown that six of the country's nine provinces—KwaZulu-Natal, Mpumalanga, Limpopo, Eastern Cape, Western Cape, and Gauteng—had infections in both animals and snail intermediate hosts (Malatji et al., 2020; Malatji and Mukaratirwa, 2019; Chikowore et al., 2019; Haridwal et al., 2021). Nucleic acid-based techniques have revealed the presence of *F. gigantica* in KwaZulu-Natal (Mucheka et al., 2015), Mpumalanga (Chikowore et al., 2019), the Eastern Cape (Malatji and Mukaratirwa et al., 2019), and *F. hepatica* in Mpumalanga (Muckeka et al., 2015), Gauteng (Mucheka et al., 2015; Haridwal et al., 2021). Studies also revealed a geographical overlap in the distribution of the two species in KwaZulu-Natal and Mpumalanga provinces (Malatji et al., 2020; Chikowore et al., 2019; Haridwal et al., 2021; Mucheka et al., 2015). The genetic diversity and intermediate forms of the two *Fasciola* spp. in South Africa are still poorly understood, and there is currently no knowledge of the existence and sequence variation of the two *Fasciola* spp. in the North West Province.

Internal transcribed spacer 2 (ITS-2) and cytochrome C oxidase subunit I gene (COI) sequences were used to determined the phylogenetic relationship of *Fasciola* species (Omar et al., 2013). Although the CO1 mitochondrial marker is highly variable and hence viewed as more effective in resolving taxonomic patterns of *Fasciola* and closely related species, the ITS nuclear marker can be employed for species differentiation (Semyenova et al., 2005; Mucheka et al. 2015; Patwardhan *et al*., 2014). Mucheka *et al*., (2015) used the *ITS-1*. *Fasciola* spp. were identified in South Africa (KwaZulu-Natal and Mpumalanga) and Zimbabwe using the ITS-2 and COI genes as identifiers. In a different study, Chikowore *et al*., (2019) used CO1 sequences to distinguish between *Fasciola* spp. in three abattoirs in the South African provinces of Mpumalanga and

KwaZulu-Natal. They found that all isolates from KwaZulu-Natal identified as *F. hepatica*, while isolates from Mpumalanga Province contained both *F. hepatica* and *F. gigantica* (Chikowore *et al*., 2019). Using the CO1 and ITS-1/5.8S/ITS-2 markers, the identities of *F. hepatica* and *F. gigantica* were recently confirmed in two abattoirs in Mpumalanga, but only *F. hepatica* in one abattoir in KwaZulu-Natal (Haridwal *et al*., 2021). There are numerous instances of intermediate forms of *Fasciola* species that may be difficult to differentiate morphologically but may be discriminated using ITS sequence data (Itagaki and Tsutsumi, 1998; Itagaki *et al*., 1998; Schweizer *et al.*, 2007). (Itagaki *et al*., 2009). There is a need for greater research on the occurrence and evolutionary links of the two species because of the changing epidemiological state of *Fasciola*.

The aim of this study was to identify the DNA of *Fasciola* species from faecal samples of cattle reared communally by smallholder farmers in the North West Province, South Africa, and establish their phylogenetic relationships using the nuclear ribosomal *ITS-2* and mitochondrial CO1 sequences. The findings can be used to create awareness amongst stakeholders, including policymakers, about the risk to livestock and public health of fasciolosis.

6.2 Materials and methods

6.2.1 Description of the study area

The North West province is one of the nine provinces of South Africa, located in the northcentral area of the country. This province is the fourth largest beef cattle producer with 1,576 head of cattle, which is 12.9% of the total national cattle population of 12,234 (National Livestock Statistics, DAFF 2021). Smallholder farmers in this province typically raise cattle under an extensive management system. This study was conducted in three villages (Mkapanstad, Ga-Motle, and Tladistad) in the Moretele Local Municipality, falling under Bojanala District

Municipality in the province. Makapanstad is located at 25° 14' 36" South and 28° 7' 19" East with a total area of 20.45 km^2 and a human population just above 15000. Ga-Motle is located at 25° 21' 14" South and 28° 4' 9" East covering an area of 8.3 km^2 with a human population of 5600, while Tladistad is located at 25° 12′ 10.8″ South and 28° 2′ 6″ East, with an area of 3.30 km^2 and a human population of just above 3000. Annual rainfall is approximately 360mm with much of the rains falling within the summer months of October through April. The temperatures range from 3° to 21 °C in winter and from 17° to 31 °C in the summer.

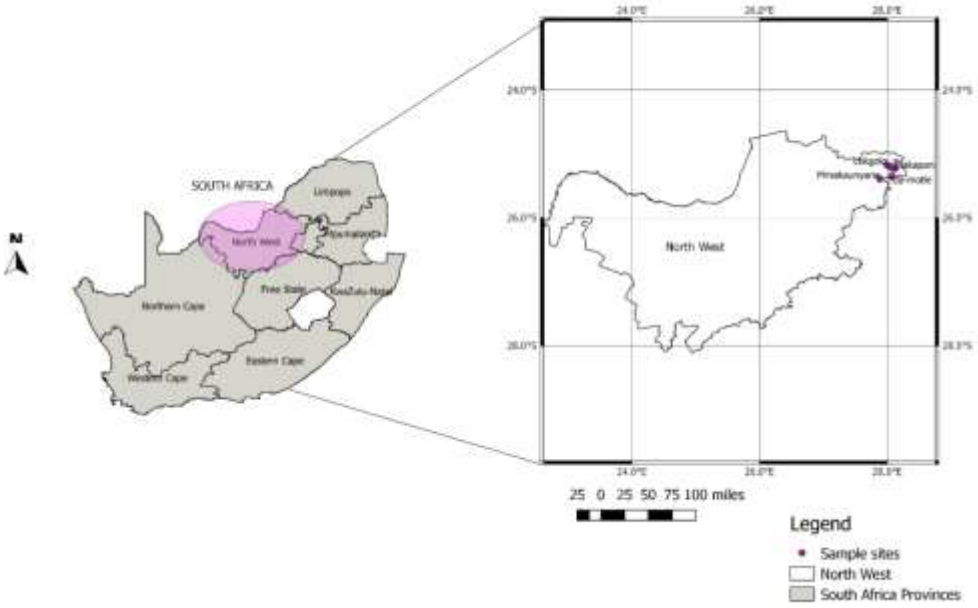

Figure 6.1: Map of the research area, the Moratelle municipality area in South Africa's North West Province. A map of South Africa with the location of the North West Province is included. The sample locations for this study are represented by the pink dots in the Moratelle municipality region. The map was created using the QGIS software version 2.8.

6.2.2 Study design, sample size, sample collection, and DNA extraction

The procedure adopted the systematic random sampling technique. The animals were of different breeds, sex, and age. Feacal samples were collected from the rectum of each animal into 80-ml plastic faecal containers using a lubricated gloved hand, The containers were subsequently labeled with the village name, date of sampling, breed, sex, age, and faecal score. The collected samples were placed in a cooler box with ice packs and then transported to the Parasitology Laboratory in the Department of Veterinary Tropical Diseases at the University of Pretoria, South Africa for further analysis.

The sample size was estimated based on the formula by Thrusfield (2018) as follows:

$$n = 1.962\frac{Pexp(1 - Pexp)}{d^2}$$

Where: n= total number of sample size; d= absolute precision (5%); Pexp = expected prevalence. An estimated prevalence of 23.3% was used based on a previous study on the detection of *Fasciola* spp. using post-mortem liver inspection at abattoirs in the Eastern Cape province (Jaja et al., 2017). The calculated sample size was 275 cattle.

6.2.3 Sedimentation technique to identify fluke eggs

Eggs of *Fasciola* spp. were identified and enumerated using sedimentation technique as previously described (Calvani *et al.,* 2017), with some modifications.

6.2.4 DNA extraction

With minimal modifications, the QIAamp ® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to extract DNA from the faecal sediments (n=277) (Calvani et al., 2017). A 15-ml plastic cylindrical tube containing the faecal material was filled, and it was centrifuged at

4,000 rpm for 40 minutes. In a pre-made tube containing ceramic beads (MagNA Lyser Green Beads, Roche Diagnostics, Mannheim, Germany) and 700 µl of lysis buffer, AL, the pellet was collected using a sterile fine wooden applicator stick (QIAGEN, Hilden, Germany). The mixture was homogenised at 6800 revolutions per second for 36 seconds in a Precellys 24 Tissue Homogeniser (Bertin Technologies SAS, Montigny-le-Bretonneux, France). This was followed by incubation at 85 °C for 10 min. About 600 µl of the mixture was transferred into a 2-ml microcentrifuge tube containing 25 µl proteinase K, followed by the addition of 700 µl of InhibEx from the extraction kit. The mixture was vortexed for 15 s and then incubated at 70 °C for 24 h. The rest of the DNA extraction procedure, starting with the addition of ethanol, was as described in the QIAamp ® Fast DNA Stool Mini Kit protocol. The extracted DNA was stored at -20°C until further analysis.

6.2.5 *Fasciola* quantitative real-time PCR

A previously published qPCR assay (Alasaad *et al*., 2011) was used to amplify a 140-base pair (bp) fragment of the *Fasciola* internal transcribed spacer 2 (*ITS-2*) gene from collected faecal samples. The PCR primers and probes as well as the procedure were based on previous protocols (Alasaad *et al*., 2011) with some modifications. The *F. hepatica* (ProFh) and *F. gigantica* (ProFg) probes were instead labeled with FAM or VIC reporter dyes, respectively, at the 5'ends, and each probe was labeled with a QSY quencher dye at the 3'-end. Each PCR reaction contained 2 µl of DNA template, 0.3 M of each primer, 0.1 M of the FAM and VIC-labelled probes, and 1X TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa) in a total reaction volume of 20 µl. In a StepOnePlusTM Real-Time PCR System (Applied Biosystems, Life Technologies, Johannesburg, South Africa), thermal cycling was

carried out under the following circumstances: AmpliTaq Gold pre-activation at 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s and annealing at 60 °C for 1 min, all took place after uracil N-glycosylase digestion at 50 °C for 2 minutes. Positive controls were DNA previously extracted from adult *Fasciola* worms obtained from infected livers, confirmed using PCR and DNA sequencing. The negative control was nuclease-free water.

6.2.6 *Fasciola ITS-2* and CO1 PCR

Fasciola hepatica and *F. gigantica ITS-2* sequences were obtained by amplifying the ~364-bp and ~300-bp genes, respectively, using species-specific conventional PCR assays, followed by cloning and sequencing. The *Fasciola* genus-specific CO1 sequences were obtained by amplifying the ~497-bp region using a genus-specific PCR assay.

This PCR was performed on all samples with positive amplification on the *Fasciola* qPCR assay. The *F. hepatica ITS-2* gene was amplified using primers Fh-ITS-2F (5'- GTT ATA AAC TAT CA CGA CGC CCA AA-3') and Fh-ITS-2R (5'- GAA GAC AGA CCA CGA AGG GTA-3') (Kim *et al*., 2014; Lee *et al*., 2017), while the *F. gigantica ITS-2* gene was amplified using primers Fg-ITS-2F (5'- TAT CAC GAC GCC CAA AAA GT -3') and Fg-ITS-2R (5'- CCA AGT TCA GCA TCA AAC CA -3') (Lee *et al*., 2017). PCR conditions were as described previously (Kim *et al*., 2014; Lee *et al*., 2017), with some modifications. The reaction mixtures contained 2.5 µl of template DNA, 0.5 M of each primer, 1X PhusionTM Flash High-Fidelity PCR Master Mix (Thermo ScientificTM, LTC Tech South Africa [Pty] Ltd, Randburg, South Africa), and nuclease-free water in a total volume of 25 µl. A denaturation stage at 98°C for 10 s, 35 cycles at 98°C for 1 s, 60°C for 5 s, and 72°C for 15 s, and a final extension at 72°C for 1 min made up the amplification cycles.

The primers FHCO1 (forward: 5’ -TTG GTT TTT TGG GCA TCC T-3’) and FHCO1 (reverse: 5’ –AGG CCA CCA CCA AAT AAA AGA-3’) (Mucheka et al., 2015) were used to amplify the CO1 region. With a few adjustments, the PCR conditions were those previously described (Mucheka et al., 2015). The reaction mixtures contained 2.5 µl of template DNA, 0.4 M of each primer, 1X PhusionTM Flash High-Fidelity PCR Master Mix (Thermo ScientificTM, LTC Tech South Africa [Pty] Ltd, Randburg, South Africa), and nuclease-free water in a total volume of 25 µl . Thermo Scientific's Veriti 96-Well Fast Thermal Cycler was used to conduct the PCR, which was done under the following conditions: initial denaturation at 98°C for 10 s, 30 cycles of 98°C for 1 s, 59°C for 7 s, 72°C for 15 s, and a final extension at 72°C for 1 min.

As a negative control, PCR-grade water was added to each run, and as a positive control, DNA retrieved from adult Fasciola was obtained from slaughter calves and verified by morphological identification and DNA sequencing. By electrophoresis on 2% TAE agarose gels stained with ethidium bromide, the PCR results were examined.

6.2.7 Cloning and sequencing of the ITS-2 and CO1 gene

Eight (six positives for both *F. hepatica* and *F. gigantica* assays; two positives for *F. hepatica* PCR only) were randomly selected for cloning and sequencing out of all positive samples. For the CO1 region, amplicons from all 12 *Fasciola* PCR-positive samples were cloned and sequenced. The PCR products were purified using the QIAquick PCR Purification Kit from QIAGEN, Hilden, Germany, and then ligated into the pJET vector using the CloneJET® PCR Cloning Kit from Thermo Scientific, LTC Tech South Africa [Pty] Ltd, Randburg, South Africa. These steps resulted in the transformation of *Escherichia coli* HST08 strain cells (StellarTM Competent Cells, Takara

Bio USA, Inc., San Jose, CA, United States). From each sample, four to ten recombinant plasmid clones were randomly chosen and examined using colony PCR in the manner previously reported by (Byaruhanga *et al*., 2018). In a total volume of 20 µl, each PCR reaction mixture contained 1x DreamTaq Green PCR Master Mix (Thermo ScientificTM, Randburg, South Africa), 0.2 µl of each of the vector primers pJET1.2F and pJET1.2R, one colony as a template, and nuclease-free water. Using the primers pJET1.2F and pJET1.2R, amplicons from the recombinant clones were sequenced at the Inqaba Biotec sequencing facility (Inqaba Biotechnical Industries [Pty] Ltd, Pretoria, South Africa) to ensure that they were the correct size (364 bp for *F. hepatica* ITS-2, 300 bp for *F. gigantica* ITS-2, and roughly 497 bp for Fasciola CO1.

6.2.8 ITS-2 and C01 sequence analysis

CLC Genomics Workbench version 7.5.1 (CLC Bio, Boston, MA, United States of America) was used to process the ITS-2 and CO1 clone sequences before sending them to the Basic Local Alignment Search Tool [BLAST] to find related reference sequences. Using Multiple Alignment with the Fast Fourier Transform (MAFFT) version 7.0, the sequences were aligned with published sequences from GenBank (Katoh and Standley, 2013). Molecular Evolutionary Genetics Analysis [MEGA] version 11.0 was used to do pairwise comparisons to determine genetic distances (% identity and number of nucleotides) (Tamura *et al.*, 2021).

6.2.9 Phylogenetic analysis

Based on the Akaike Information Criterion (AIC), jModelTest version 2.1.3 was used to find the best-fit nucleotide substitution models, TPM3uf and TPM3uf+I, for the Fasciola ITS-2 and CO1 sequences (Darriba et al., 2012). PhyML 3.1, which uses a maximum likelihood approach, was used to reconstruct phylogenetic trees using the ITS-2 and CO1 reference and newly discovered

sequences (Guindon et al., 2010). As previously indicated, 1000 replicates were used to assess the dependability of the internal branches of the trees (Felsenstein, 1985). Phylogenetic trees were graphically shown and edited using MEGA11 and Paint Tool for Windows 10.0.

6.2.10 Nucleotide sequence accession numbers

The sequences for the ITS-2 gene from *F. hepatica* and the 10 CO1 gene from *F. gigantica* were deposited in the GenBank database with accession numbers ranging from ON721137 to ON721151 and OP265009-OP265018, respectively.

6.3 Results

6.3.1 *Fasciola* ITS-2 and CO1 amplicons

Seventy-three (26.4%) of the samples were qPCR positive and tested using the *Fasciola* ITS-2 species-specific and *Fasciola* genus-specific CO1 conventional PCR assays. Twelve (16%) samples were positive for *F. hepatica* ITS-2 only and 7 (9.6%) for *F. gigantica* ITS-2 only. Positive samples were from the five different locations (Table 6.1). Six *F. gigantica* ITS-2 (~300 bp) sequences were identified by BLAST in homology search from six clones from three samples (97, 201, 230), while nine *F. hepatica* ITS-*2* sequences (~364 bp) were identified from nine clones from five samples.

Fasciola sequences were obtained from 10 out of the 12 samples subjected to CO1 gene amplification, cloning, and sequencing. One sample was not successfully cloned and another sample produced only cloning vector sequences. Clone sequences from six samples corresponded to *F. hepatica*, while clone sequences from four samples corresponded to *F. gigantica*.

Three and two samples yielded *F. hepatica* and *F. gigantica* sequences respectively, with both CO1 and ITS-2 regions. However, sample numbers 185 and 230 yielded *F. gigantica* and *F. hepatica* sequences respectively with the CO1 gene, these two samples yielded F.hepatica alone with the ITS-2 gene.

Table 6.1: *Fasciola* species-specific ITS-2 conventional PCR, cloning, and sequencing results of bovine faecal samples collected from farms in the North West Province, South Africa

Sample	Location	Conventional ITS-2 PCR results	Clone	Sequence classification	Accession number
9	Legkolo	F. hep, F. gig	9.1, 9.2	*F. hepatica*	ON721139, ON721140
97	Motla-Bosawala	F. hep, F. gig	97.1, 97.6	*F. gigantica*	ON721137, ON721138
168	Makayauna	F. hep	168.1, 168.3	*F. hepatica*	ON721141, ON721142
185	Nrokie	F. hep, F. gig	185.1, 185.4	*F. hepatica*	ON721143, ON721144
201	Nrokie	F. hep, F. gig	201.1, 201.5	*F. gigantica*	ON721147, ON721148
230	Nrokie	F. hep, F. gig	230.3, 230.4	*F. gigantica*	ON721145, ON721146
257	Kromkail	F. hep, F. gig	257.3, 257.6	*F. hepatica*	ON721149, ON721150
259	Kromkail	F. hep	259.1	*F. hepatica*	ON721151

The eight samples were randomly selected from a list of 73 samples that had tested positive *Fasciola* species using a real-time PCR assay. *F. hep*, *Fasciola hepatica*; *F. gig*, *Fasciola gigantica.* Clone size: 364 bp – *F. hepatica*; 300 bp – *F. gigantica*.

Table 6.2: *Fasciola* genus-specific CO1 conventional PCR, cloning, and sequencing results of bovine faecal samples collected from farms in the North West Province, South Africa

Sample	Location	Clone	Sequence classification	Accession number

9	Legkolo	9.1	*F. hepatica*	OP265009
97	Motla-Bosawala	97.9	*F. gigantica*	OP265010
185	Nrokie	185.4	*F. gigantica*	OP265011
201	Nrokie	201.4	*F. gigantica*	OP265012
230	Nrokie	230.1	*F. hepatica*	OP265013
257	Kromkail	257.1	*F. hepatica*	OP265014
259	Kromkail	259.1	*F. hepatica*	OP265015
165	Makayauna	165.3	*F. hepatica*	OP265016
51	Motla-Bosawala	51.1	*F. hepatica*	OP265017
258	Kromkail	258.1	*F. gigantica*	OP265018

The eight samples were randomly selected from a list of 73 samples that had tested positive *Fasciola* species using a real-time PCR assay. F. hep, *Fasciola hepatica*; F. gig, *Fasciola gigantica.* Clone size: 364 bp – *F. hepatica*; 300 bp – *F. gigantica*.

6.3.2 Sequence analysis

Fifteen *F. hepatica* and nine *F. gigantica* ITS-2 sequences were obtained in this study. *Fasciola hepatica* and *F. gigantica* ITS-2 sequences were identical within species but varied at 3 to 4 nucleotide positions across species. Ten *Fasciola* CO1 sequences were obtained, with all four *F. gigantica* CO1 sequences identical to each other. Of the six *F. hepatica* CO1 sequences, four were identical but differed from the two other sequences at four base positions. The *F. hepatica* CO1 sequences differed from *F. gigantica* sequences at 25 to 29 base positions. Four *F. hepatica* ITS-2 sequences (GenBank accession numbers ON721139, ON721140, ON721143, and ON721144) from two samples had 99.7% identity (query cover 100%, over a length of 364 bp) with a *F. hepatica* sequence identified from cattle in Mexico (MG569983) and 100% identical to two *F. hepatica* sequences from sheep in China (MH385388) and Libya (MT025436). The other five *F. hepatica ITS-2* sequences (ON721141, ON721142, ON721149, ON721150, and ON721151) from three samples were 100% identical (query cover 100%, over a length of 364 bp) to an *F. hepatica*

sequence from cattle in Spain (AJ272053). Six *F. gigantica* ITS-2 sequences (ON721137, ON721138, ON721147, ON721148, ON721145, and ON721146) from three samples were 100% identical (query cover 100%, over a length of 300 bp) to *F. gigantica* sequences from sheep and cattle in Libya (MT025356) and Egypt (MT423006), respectively.

The *F. gigantica* CO1 sequences were 96.9% (query cover 100%) identical to a sequence from the whole genome of *F. gigantica* from China (MH621335), 98.2% identical; query cover 89%, over a length of 497 bp) to an *F. gigantica* sequence from cattle in Nigeria (MW258702) and 100% (query cover 76%, over a length of 497 bp) identical to a sequence from Zimbabwe (KT182288). *Fasciola hepatica* CO1 sequences were 99.2 to 100% identical (query cover 100%) to a sequence from the whole genome of *F. hepatica* from Japan (AP017707), 99.2% to 100% (query cover 99%) identical to *F. hepatica* sequence from cattle in Austria (MN507459) and 99.2 to 99.7% identical (query cover 76%) to a sequence from Zimbabwe (KT182306).

6.3.3 Phylogenetic analysis

i sequences from each sample were identical; therefore, one representative sequence for each sample was included in the phylogenetic analysis. The newly-identified *F. hepatica* ITS-2 sequences were grouped into two different clusters, one cluster (clade 2) with reference sequences from China, Libya, and Peru and the other cluster (clade 3) with a sequence from Spain (Figure 6.2). The *F. gigantica* ITS-2 sequences were grouped in one cluster together with sequences from sheep from Libya (MT025356) and cattle from Chad (MK321625) (Figure 6.2).

Of the 10 *Fasciola* CO1 sequences, one sequence (257.1) was relatively short (437 bp) and therefore not included in the phylogenetic tree. Four *F. gigantica* sequences grouped with *F.*

gigantica isolate sequences from Zimbabwe, while five *F. hepatica* sequences grouped with *F. hepatica* sequences from sheep and cattle from Japan, Tunisia, Austria, and Ecuador (Figure 6.3).

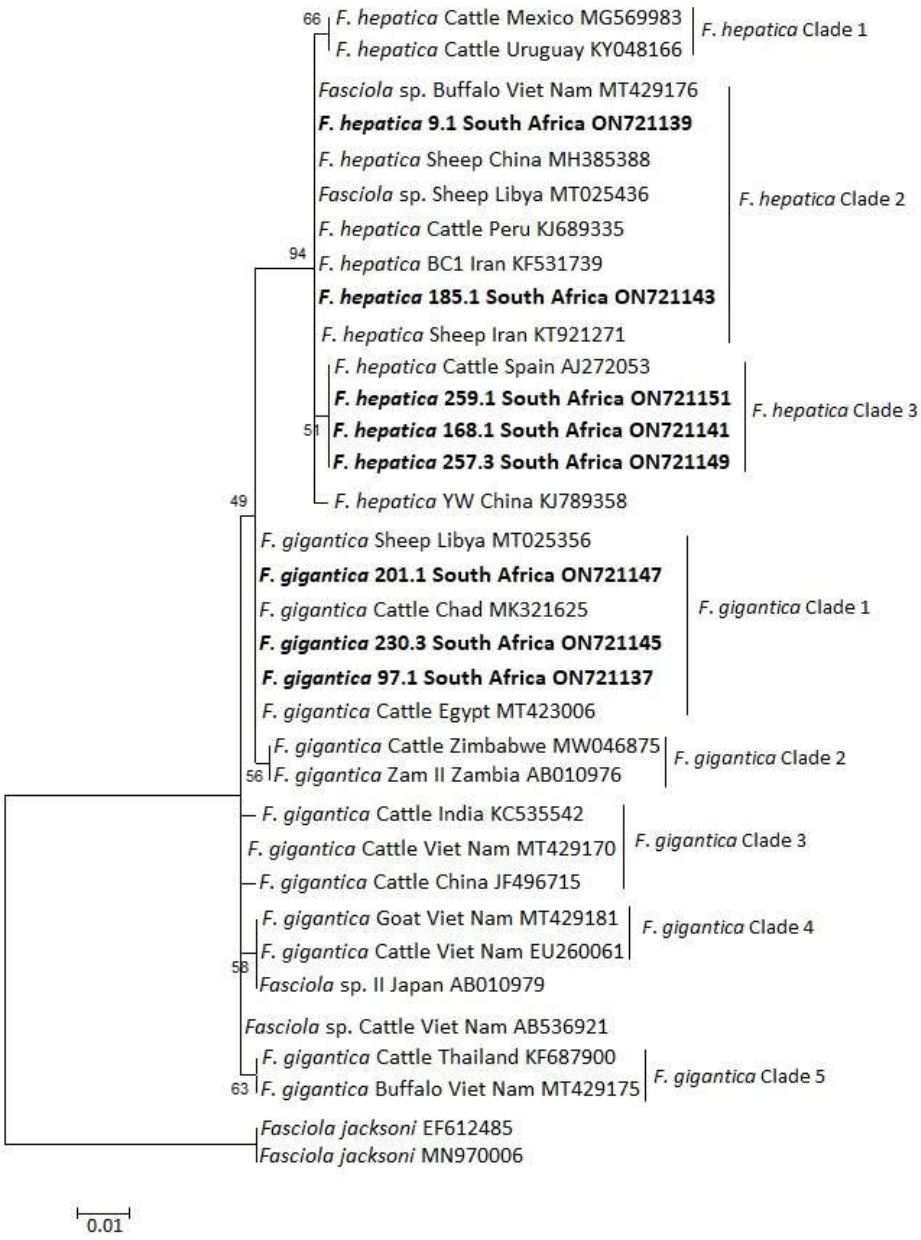

Figure 6.2: Tree showing relationship of internal transcribed spacer 2 (ITS-2) nucleotide sequences of *Fasciola* DNAfrom cattle feacal samples in North West province, South Africa (in bold) after being analyzed phylogenetically with references from other countries. The maximum likelihood approach was used to conduct the analysis. The percentage of the 1000 repeats (bootstrap) for which the identical branching patterns were obtained is shown by the numbers at the internal nodes. *Fasciola jacksoni* ITS-2 nucleotide sequences isolated from an Asian elephant in Sri Lankan were used to establish the tree's roots. Each sequence's accession number is listed next to the sequence name. Branch lengths are inversely correlated with the estimated genetic distance between taxa (measured as the average number of nucleotide substitutions per site over the ITS-2 nucleotide sequence's 367 locations).

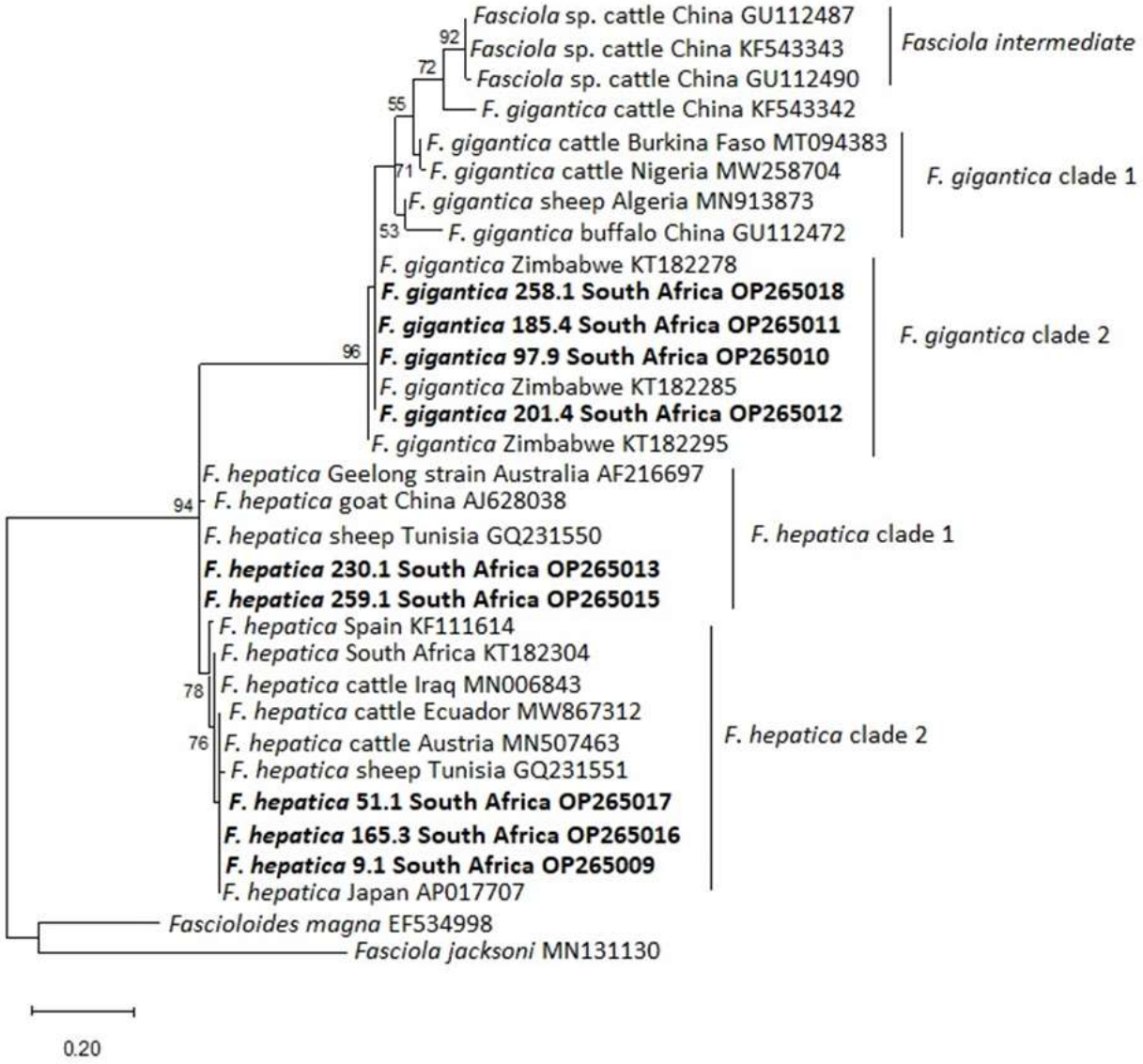

Figure 6.3: *Fasciola* cytochrome c oxidase subunit 1 (CO1) nucleotide sequences from cattle in the North West Province of South Africa (in bold) after phylogenetic analysis using reference sequences. The maximum likelihood approach was used to conduct the analysis. The percentage of the 1000 repeats (bootstrap) for which the identical branching patterns were obtained is shown by the numbers at the internal nodes. *Fascioloides magna* and *Fasciola jacksoni* CO1 nucleotide sequences were used to establish the roots of the tree. Each sequence's accession number is listed next to the sequence name. Branch lengths are inversely correlated with the estimated genetic

distance (measured as the average number of nucleotide substitutions per site over the CO1 nucleotide (sequence's 497 locations) between the taxa.

6.4 Discussion

Both *F. hepatica* and *F. gigantica*, as well as their intermediate species, have been discovered in numerous countries, including China (Liu *et al*., 2014), Korea (Agatsuma *et al*., 2000), Vietnam (Le et al., 2008), Japan (Ichikawa & Itagaki, 2010), Northern Iran (Amor et al., 2011), Niger (Ali et al., 2008), South Africa (Muchenka *et al.*, 2015; Chikowore *et al*., 2019; Haridwal *et al*., 2021). According to earlier research, reliable genetic markers for the differentiation and identification of *Fasciola* species can be obtained by sequencing the ITS-2 and CO1 sections of these species (Farjallah et al., 2013). In this present study, we analysed sequences from the *ITS-2* and CO1 regions of *Fasciola* species in faecal samples from cattle from the North West Province, South Africa to characterise the species present in the study area. In another study, a similar model was adopted for the characterization of *Fasciola* samples in the Republic of Niger (Ali et al., 2008). Elsewhere, ITS-1 and ITS-2 analyses were employed as genetic markers for *Fasciola* species identification in Japan, Korea, and Bangladesh (Lin *et al*., 2007).

In the present study, out of the eight samples randomly selected for ITS-2 sequencing, five (62.5%) sequences corresponded to *F. hepatica* and three (37.5%) to *F. gigantica*. Out of the ten samples randomly selected for CO1 sequencing, six (60%) sequences corresponded to *F. hepatica,* and four (40%) *to F. gigantica.* Results from the North Westprovince are similar to those of Chikowore *et al*. (2019) who reported 100% of *F. hepatica CO1* in KwaZulu-Natal Province and 76% *F. hepatica*, 24% *F. gigantica* and 12% of mixed infection from the Barberton abattoir in the Belfast area of Mpumalanga Province of South Africa. The differences observed in the percentage

distribution pattern of *Fasciola* species in this study and the previous study with CO1 sequences in KwaZulu-Natal and Mpumalanga provinces might be associated with differences in transmission patterns with the occurrence of the snail intermediate hosts. In addition, Chikowore *et al*. (2019)'s study was based on CO1 mitochondrial marker, while the present study is based on the nuclear rDNA ITS2 and CO1 mitochondrial marker. This distribution is similar to what has been previously described by Mucheka *et al*. (2015) in South Africa. A relatively higher prevalence of *F. hepatica* (66.7%) compared to *F. gigantica* (33.3%) was also reported in Iran with phylogenetic alignment identifying variable sites at positions 59, 68, 186, 276, 370, 448, and 468 where single-base substitutions caused differentiation of specimens (Moghaddam *et al*., 2004).

Previous reports of mixed infections of the two species were documented in Egyptian cattle (Amer et al., 2011). However, this discovery contrasts with earlier research (Chaudhry et al., 2016), which found that all isolates were *F. gigantica* and that *F. hepatica*, *F. gigantica*, and even both co-existed in a small number of the animals analyzed. The current study is based on nuclear rDNA ITS2 and COI mitochondrial marker, whereas the previous study (Chaudhry et al., 2016) was based on rDNA ITS-2 gene sequences. Environmental conditions and host-related aspects may have an impact on the distribution of *Fasciola* species. Both species of *Fasciola* intermediate hosts are freshwater lymnaeid snails (Thanh, 2012). While the intermediate host of *F. gigantica, R. natalensis* (Walker et al. 2008) is typically found in the tropical and subtropical regions of Africa, *G. truncatula* serves as the intermediate host for *F. hepatica*, which is more prevalent in Europe, America, and Asia. The five snail species that have been recorded in South Africa are *Radix auricularia* (), *G. truncatula*), *P. columella*, *Radix rubiginosa*, and *R. natalensis*. *Radix natalensis*,

P. columella, and *G. truncatula* occur in the North West Province (Nyagura et al., 2022) and this could explain the occurrence of both *Fasciola hepatica* and *gigantica* species in the study area. With the CO1 gene, two samples, 185 and 230, respectively produced sequences from *F. gigantica* and *F. hepatica*, whereas this was the opposite with the ITS-2 gene. Within the same fluke, sequence polymorphism has been seen across copies of the ITS-2 array, with one copy having *F. hepatica*-like sequences and the other nearly identical to *F. gigantica* sequence (Huang et al., 2004). The other possibility is the presence of eggs of both *Fasciola* spp. in the same sample, with the resultant amplification of different species for the CO1 and ITS-2 PCR assays. However, the adoption of the COI gene for the current study verifies the identity of the *Fasciola* species present because it is a more effective marker for genotypic identification and differentiation of *Fasciola* spp. (Mucheka et al., 2015).

The ITS-2 phylogenetic analysis identified distinct clades for the two *Fasciola* species which indicates the dissimilarity between the two species. The phylogenetic analysis of *Fasciola* internal transcribed spacer *2* (ITS-2) nucleotide sequences of *F. hepatica* and *F. gigantica* in this present study are similar to previous reports (Giovanoli Evack et al., 2020). The discovery of both *Fasciola* species and even mixed infections is also consistent with earlier report in the South African provinces of KwaZulu-Natal (Eshowe) and Mpumalanga (Ehlanzeni) (Mucheka et al. 2015). Ethiopia and Niger are two African nations where *Fasciola* species hybridization has been documented (Walker et al., 2008; Ali et al., 2008). *Fasciola gigantica* and *F. hepatica* hybrids could possess a more serious threat because they may be more adaptable, allowing them to expand their host and geographic ranges as well as devel op stronger anthelmintic resistance. This would represent a significant threat to both animal and human health and have disastrous repercussions

on the economies of populations with limited resources when prevention and control measures are difficult to implement.

The sparse number of available sequences and the dispersed location of the sampling locations placed restrictions on the study. The genetic diversity and population structure of *Fasciola* spp. would be better represented through molecular analysis of a large number of isolates from various host species and geographical regions, and this would also assist to clarify the dynamics of transmission in South Africa. This study has, however, demonstrated infection with both *Fasciola* species in cattle in the North West Province which has significant ramifications for understanding species distribution and disease control in the province.

6.5 Conclusion

To the best of our knowledge, this study is the first on *Fasciola* species among communally reared cattle in the North West Province of South Africa, adding to existing knowledge of the species prevalence and genotypic makeup in the study region. The identification of mixed infections might indicate future *Fasciola* species hybridization in the study area. We found sequence variation in *Fasciola* spp., which is similar to previous findings from other African countries (e.g. Egypt, Libya, and Chad) and elsewhere in the world (e.g. (Mexico and Spain). Appropriate fasciolosis control strategies are recommended in the communal farming areas of North West Province.

6.6 References

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CHAPTER 7: General Discussion, Conclusions, and Recommendations

7.1 General Discussion

Fasciolosis is an economically important parasitic and zoonotic disease of domestic ruminants and humans worldwide due to mortality, liver condemnation, productivity losses (meat, milk, and drought power), reproductive failure, infertility, and frequent treatment costs. Jaja *et al.* (2017) reported that prevalence ranged from 2% to 14.4% based on a retrospective study and from 24.5% to 53.1% based on a prospective study in three different abattoirs in one province of South Africa. However, data on the knowledge of cattle farmers concerning the epidemiology of bovine fasciolosis are scant. Additionally, the prevalence of the disease in cattle and associated haematological and biochemical changes in infected communal cattle remain undetermined. Lastly, the *Fasciola* species infecting cattle in the communal grazing areas of North West province are unknown. The main hypothesis tested in the present study was that there is a low level of knowledge and, poor practices of smallholder farmers about bovine fasciolosis, low prevalence, and that there will not be *Fasciola* species genotypic differences in the communal grazing areas of the North West province.

Inadequate knowledge of the disease, the presence of multiple high-risk farm practices, inappropriate perceptions, and bad practices require education for improvement. It is, therefore, imperative to start by assessing the smallholder farmers' level of knowledge, attitude, and practices about bovine fasciolosis, as this is essential for the institution of prevention and control strategies. In Chapter 3, smallholder cattle farmers' knowledge, attitudes, and practices regarding the risk factors, zoonotic importance, transmission, prevention, and control of bovine fasciolosis in the North-West Province of South Africa were assessed. It was hypothesized that the smallholder

cattle farmers possessed poor knowledge, attitudes, and practices on the epidemiology of bovine fasciolosis. The majority of smallholder farmers were typically males, less educated, and married with more than 10 years of cattle rearing experience; this is typical of communal farming operations in South Africa (Katikati and Fourie, 2019; Mmbengwa et al., 2015). The lack of significant predictors on epidemiological knowledge of the disease suggests that the level of knowledge in sampled communities was relatively unpredictable and that they possessed random knowledge without an adequate understanding of the epidemiology of the disease. This could also imply a general lack of training, absence of training materials, and very few people possessing knowledge in the study area. Highly educated farmers, and semi-intensive practicing farmers were more likely to possess positive practices to prevent and control bovine fasciolosis than the less educated farmers and those practicing extensive or backyard operations.

In Chapter 4, the prevalence of fasciolosis in naturally infected cattle reared communally by smallholder farmers in the North West province was established using the sedimentation technique, real-time PCR, and Ag ELISA, and the detection rate of the three methods was compared. It was hypothesised that the three diagnostic techniques will give a similar bovine fasciolosis detection rate. The qPCR technique had a relatively higher specificity and sensitivity of 25% and 73.4% respectively compared to other diagnostic methods and this may be associated with the higher sensitivity of qPCR compared to other methods earlier observed by (Calvani et al., 2017). There was no detection of *Fasciola* species by coproAg ELISA technique, no agreement between qPCR and sedimentation, and a lower prevalence of bovine fasciolosis generally observed in in the present study compared to previous prevalence reports in the Eastern Cape, South Africa. The failure of Ag ELISA to detect any positive samples in the present study could be attributed to

the generally low egg counts (< 10 EPG) observed in the faecal samples, which most likely affected the sensitivity of the Ag ELISA (Brock-well et al., 2013)Martinez-Sernandez et al., 2016). Poor agreement between the qPCR and sedimentation test in the present study could be suggestive of poor accuracy of the PCR test which may be affected by PCR inhibitors in faeces which leads to false negative PCR results (Acharya et al., 2017). The prevalence of bovine fasciolosis observed in the present study was relatively higher than 5% to 23% (Jaja et al., 2017) and greatly lower than 16.4% to 73.6% (Mpisana et al., 2022) detected in the Eastern Cape Province of South Africa. Differences in the location, study types, and diagnostic tests used in these two studies could explain discrepancies with the current study.

To improve knowledge on clinical treatment and management of bovine fasciolosis, hematological and some biochemical parameters of veterinary clinical importance were determined and compared between the shedding and non-shedding cattle in Chapter 5. The hypothesis tested was that infection with *Fasciola* spp will significantly and negatively alter the haematological and biochemical parameters of cattle under smallholder farming management in the study area. Mean corpuscular haemoglobin concentration (MCHC) was significantly higher in in shedding- than in non-shedding cattle. Eggs per gram counts were significantly correlated with RBC, Hb, and PCV. There was a moderate reduction in Hb and PCV as the eggs per gram counts increased in this present study probably because of chronic liver inflammation associated with increased worm load which incites depression in the process of erythropoiesis (Lotfy *et al*. 2003). This positive and moderately significant influence of increased eggs per gram of faeces on reduction in Hb and PCV values has been previously reported by (Egbu *et al*., 2013).

In Chapter 6, *Fasciola* species eggs from faecal samples of communally grazed cattle in the North West province, South Africa, were characterised and their phylogenetic relationships established using the nuclear ribosomal *ITS-2* and mitochondrial CO1 regions. The hypothesis tested was that there would not be species differences in phylogenetic relationships using the nuclear ribosomal *ITS-2* and mitochondrial CO1 sequences. The ITS-2 PCR revealed a higher number of cattle infected with *F. hepatica* only (41.1%; 30/73), followed by *F. gigantica* only (34.2%; 25/73) and coinfections with both *Fasciola* spp (24.7%; 18/73). This result signifies the presence of intermediate host for both *Fasciola* spp and the possibility of hybridization of the two species in the study area. This distribution is similar to what had also been previously described by Mucheka *et al*. (2015) in South Africa. Moghaddam *et al*. (2004) also reported a relatively higher prevalence of *F. hepatica* (66.7%) compared to *F. gigantica* (33.3%) in Iran with phylogenetic alignment showing DNA variable sites in which nucleotides at the position of 59, 68, 186, 276, 370,448, and 468 were single-base substituted resulting in segregation of the specimens. This signifies that the Fasciola spp in this study area has a similar genotypic characteristic.

7.2 Conclusion

Smallholder cattle farmers in the North West province, South Africa possessed random and generalized knowledge of bovine fasciolosis, and they were generally lacking in epidemiological information on bovine fasciolosis. Less educated farmers and those involved predominantly in extensive systems of management had a poor likelihood of engaging in satisfactory practices of prevention and control of bovine fasciolosis. The qPCR was the most sensitive diagnostic test followed by sedimentation while the Ag ELISA failed to detect the presence of *Fasciola* spp. in the current study. There was a moderate (26.4%) prevalence of bovine fasciolosis in communally

grazed cattle in the North West province as detected by the more sensitive qPCR assay. Bovine fasciolosis was associated with increased MCHC in infected cattle but did not significantly alter the erythrogram, leucogram, and serum chemistry of communal cattle. Higher worm burden was correlated with reduced Hb and PCV of infected cattle. This study is the first to document *Fasciola* species among communally reared cattle in the North West Province of South Africa and contributed to the current knowledge regarding the occurrence and genotypic composition of *Fasciola* spp. in the study area. We found sequence variation in *Fasciola* spp. and this is similar to previous findings from other African countries (e.g. Egypt, Lybia, and Chad) and elsewhere in the world (e.g. (Mexico and Spain). The observation of mixed infection may be indicative of future *Fasciola* species hybridization in the study areas.

7.3 Recommendations

Training, re-training, enlightenment, and awareness sessions for smallholder farmers on epidemiological information should be provided. Especially information related to transmission modes, zoonotic importance, prevention, and control strategies. Need for public enlightenment on the zoonotic importance of bovine fasciolosis, especially in the communal grazing areas. Veterinary extension services should be improved upon and clear-cut national policy on prevention and control of major neglected tropical diseases in South Africa factoring communal grazing operations should be enacted and executed by the government. A rapid diagnostic kit for farm-based diagnosis of bovine fasciolosis may be a better, quicker, and more economical approach. Periodic screening of cattle and institution of prophylactic treatment and other preventive measures for animals and the environment against bovine fasciolosis in communal grazing. The moderate

effect of increased faecal egg counts with significant reduction in the values of Hb and PCV should be taken into consideration when treating a case of bovine fasciolosis.

The following aspects require further research:

1. Whole genome sequencing of *Fasciola* species to be isolated from stream/dam, grazing pastures, Lymnaeidae snail species, cattle, and humans in the study area.

2. Experimental studies are also required to have a better understanding of the dynamics of the organisms in their hosts.

3. More studies to establish the detection limit of the different diagnostic tests in natural infections are required.

4. The roles of freshwater Lymnaeidae snail species in the spread and transmission of *Fasciola* species and the exact types being transmitted should be established using the more advanced molecular technique.

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Appendix 1: Questionnaire

CONSENT

I …… hereby give my consent to participate in the research entitled:

"Assessment of the level of knowledge, attitudes and practices on bovine fasciolosis of smallholder cattle farmers in North West Province, South Africa."

I am fully aware that I will participate in an interview that will take approximately 30 minutes at my homestead. I am aware that the interviewer will record my responses on the questionnaire and these data will be stored for subsequent statistical analysis. All information that is collected as part of this study will remain confidential and personal information will not be disclosed without my permission. Please contact Dr. Olaogun Sunday Charles for further information regarding this study: **+27843759159.**

Signature of respondent_____ Date_____

Signature of Researcher_____ Date _____

Date of survey_____ Questionnaire reference number _____

Name of Location_____

GPS Coordinates: Longitude _____, _____, Latitude_____, _____

SECTION A (FARMER'S/ OWNER DEMOGRAPHY)

1. Are you the..... **Owner** ☐ **Animal attendant/caretaker** ☐
2. Gender: **M** ☐ **F** ☐
3. What was your age at last birthday (years) : **…… years**
4. What is your date of birth (day, month, year): **……**
5. What is your marital status: **Single (Never married)** ☐ **Married** ☐ **Divorced** ☐ **Widow** ☐
6. What is your level of education: **…….**
7. What is your home language: **Sepedi** ☐**Xhosa** ☐ **Zulu** ☐ **Afrikaans** ☐ **English** ☐ **Other specify**……
8. What is the size of your household? Adults: **Males** …… **Females** ….. Children: **Males** …. **Females** …..
9. How long have you been raising animals?: **…….**
10. What livestock species do you keep? (Rank 1 as the most important species).

Class	Cattle	Sheep	Goats	Chickens	Other Specify ……
Number					
Rank					

11. What are your sources of income? (Rank 1 as most important source)

Source	Rank

Livestock	
Crop	
Old age pension	
Salary/wages	
Other (specify)…….	
Not applicable	

SECTION B (HERD/FARM DEMOGRAPHY)

12. How many herd/herds do you have at present?: **…….**

13. What is the number of your cattle at present?: **…….**

14. What is the average age of your cattle? **……**

15. What breed of cattle do you keep? (Rank 1-5 based on number).

Type	Brahman	Nguni	Bonsmara	Non-descript	Other Specify ……
Rank					

16. What are the sexes of your cattle : (Rank 1-2 based on number)

Sex	Male	Castrated male	Female
Number			
Rank			

17. What is the body condition score of your typical cattle today (on a 5 point scale)?: **….**

18. Do you have calving pens: **Yes** ☐ **No** ☐

19. Do you have restraining equipment : **Yes** ☐ **No** ☐

20. Do you have weight estimation equipment : **Yes** ☐ **No** ☐

21. Which system of management do you adopt in your farm:

Rural Household/Backyard ☐ **Extensive** ☐ **Semi-Intensive** ☐ **Intensive** ☐

22. What is your cattle's major source of water? (Rank 1-5 based on importance)

Water sources	Stream	Public Water	Well	Borehole	Other Specify ……
Rank					

23. What is your major source of cattle feed?: (Rank 1-4 based on importance).

Feed sources	Concentrate	Grazing grass	Mixed Feeding	Other Specify ……
Rank				

SECTION C (RISK FACTORS AND AWARENESS)

C1: Risk factors and Knowledge/ level of awareness

24. How frequently do you see the following disease syndrome in your animals per year?

	never	1/yr	2-4/yr	5-10/yr	More than 10
Weight loss					
Anaemia					
Icterus/yellowish mucous membrane					
Weakness					
Abortion/					
Neonatal mortality					
Infertility					
Sudden death					
Diarrhoea					
Nasal discharge/cough					

Bottle jaw/brisket oedema					
Poor growth					
Reduced milk production					
Parasites/worms in faeces					
External parasites					

C2: Awareness about fasciolosis mode of transmission.

25. Have you heard about liver fluke (fascioliosis)?: **Yes** ☐ **No** ☐ **Unsure** ☐

26. Can you give the local name for the condition: **Yes** ☐ **No** ☐ **Unsure** ☐

27. If yes, what name is it called in your vernacular language?**.....**

28. Have you observed this disease in your herd during the last year? **Yes** ☐ **No** ☐ **Unsure** ☐

29. Have you sold or slaughtered cattle because of this disease during the last year?

Yes ☐ **No** ☐ **Unsure** ☐

30. Has any of your cattle died from this disease during the last year? **Yes** ☐ **No** ☐ **Unsure** ☐

31. What proportion of your cattle has been affected by liver fluke in your herd within the last year?:**1-5%** ☐ **5-10%** ☐ **10-15%** ☐ **15-20%** ☐ **20 - 25%** ☐

32. How is this disease spread or contacted within your herd?

	Yes	No	Unsure
Contaminated drinking water			
Grazing wet pasture			
Contact with other cattle			
Snails			
Contact with wildlife			

33. How is this disease transmitted to human?

	Yes	No	Unsure
Ingestion of undercooked liver			
Ingestion of contaminated vegetables			
Consumption of undercooked snails			
Drinking of contaminated water			

C3: Farmers' level of knowledge/awareness about some risk factors.

34. Which of the following conditions are risk factors for fasciolosis?

	Yes	No	Unsure
Prolonged wet season			
Warm summer weather			

High rainfall			
Unhygienic water trough			
High animal density			
Prolonged drought			
Early morning grazing			

SECTION D (Attitude and drugs usage practices about prevention and management of bovine fascioliosis)

35.Which of the following are measures to prevent and control the disease in your herd.

	Yes	No	Unsure
Vaccination			
Dipping/spraying			
Drenching with antihelminthic			
Composting of dung to be used as fertilizer for at least one month			
Avoid grazing waterlogged pasture			
Snail habitat management with chemicals			
Rotational grazing			
Monitoring of animals for infection			
Drainage control			

Fencing off snail habitat			

36. Please rank the effectiveness of the following products: 1-4 or 5 (4 or 5 being the most effective)

Treatment	Albendazole	Ivermectin	Triclabendazole	Flukesole	Other Specify ……
Rank					

37. How should the top ranked product be administered? **……..**

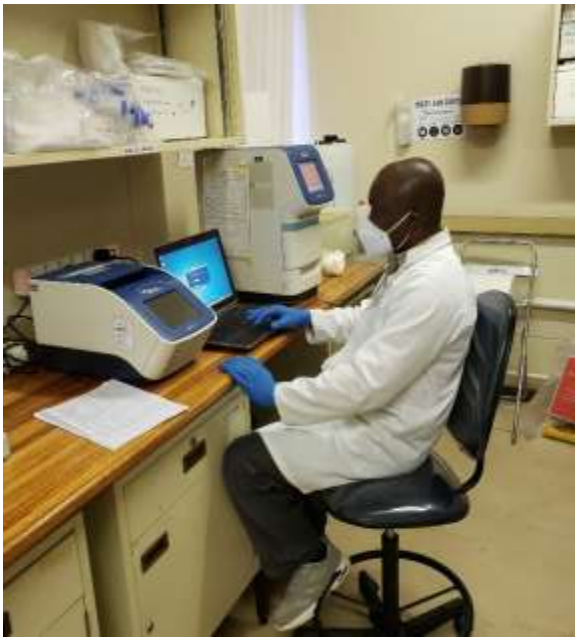
38.How many times do you administer anthelminthics before recovery? **……**

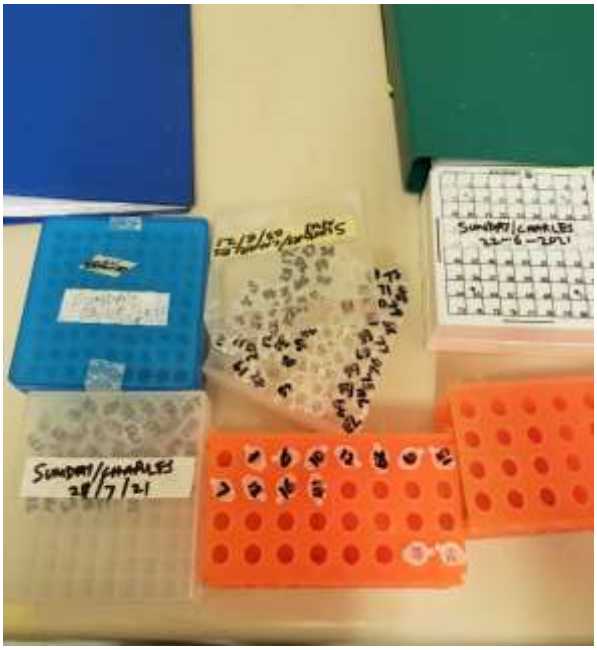
39.Is there the possibility of re-occurrence after successful recovery:

Yes ☐ **No** ☐**Unsure** ☐

Thank you for your time and efforts in filling this questionnaire.

Appendix 2: Pictures showing various stages of sample analysis

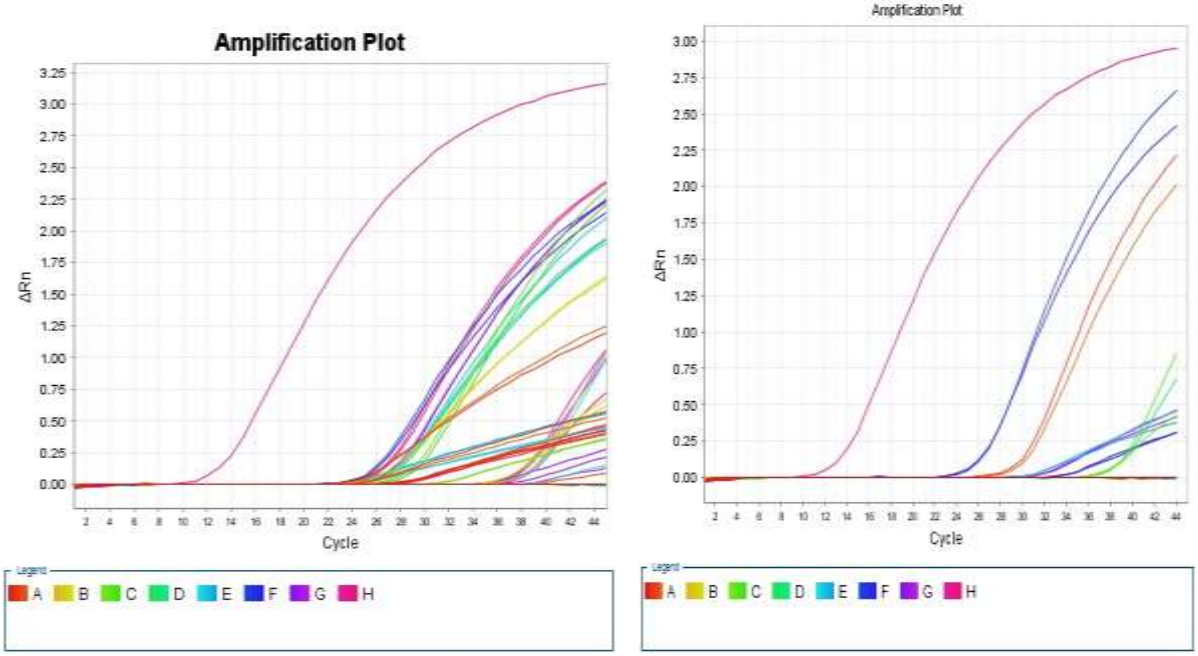





QIAamp® Fast DNA Stool
Mini Kit (50)
QIAGEN
Buffer AW1
Wash buffer 1
Buffer AW2
Wash buffer 2

Appendix 3: *Fasciola* eggs (85 x 190 µm) at 100x magnification

Appendix 4: Representative amplification plots of TaqMan qPCR showing *Fasciola* spp positivity.



Appendix 5: Ethical clearances

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee

Project Title	Molecular epidemiology of bovine fasciolosis in smallholder cattle farms in North West Province of South Africa
Project Number	REC158-19
Researcher / Principal Investigator	Dr SC Olaogun

Dissertation / Thesis submitted for	Doctoral

Supervisor	Dr MC Marufu

APPROVED	Date: 2019-11-05
CHAIRMAN: UP Research Ethics Committee	Signature:

Office of the Chairman: Research Ethics Committee
Room 2-24, Pathology Building, Onderstepoort
University of Pretoria, Private Bag X04
Onderstepoort 0110, South Africa
Tel +27 (0)12 529 8052
Email
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

4 December 2019

Approval Certificate
New Application

AEC Reference No.: REC158-19
Title: Molecular epidemiology of bovine fasciolosis in smallholder cattle farms in North West Province of South Africa
Researcher: Dr SC Olaogun
Student's Supervisor: Dr MC Marufu

Dear Dr SC Olaogun,

The **New Application** as supported by documents received between 2019-07-04 and 2019-11-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-11-25.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Cattle (Bovine)	275 max
Samples	
Faecal	275
Blood collection	6 ml per animal

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-12-04.

3. Please remember to use your protocol number (REC158-19) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/18

Dr Munyaradzi Christopher Marufu
Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Dear Dr Marufu,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 31 May 2019, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Ethics approval must be obtained prior to the start of the study;
4. Blood and faecal samples may only be obtained from cattle within the Moretele Municipality, North West Province;
5. Prior to the collection of the samples, written confirmation must be obtained from the responsible state veterinarian, Dr T Mlilo, that the farm(s) are still not under any restriction for disease control purposes;
6. Samples must be packaged in triple packaging in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No. 93 of 1996) for transportation from the farms of origin to the Faculty of Veterinary Science;
7. At the end of the study the samples must be autoclaved and disposed of by the Waste Group;
8. This Section 20 approval is valid until 1 September 2021.

Title of research/study: Molecular epidemiology of bovine fasciolosis in smallholder cattle farming areas of North West Province, South Africa
Researcher (s): Dr Munyaradzi Christopher Marufu

Institution: Faculty of Veterinary Science, University of Pretoria
Your Ref./ Project Number: REC086-19
Our ref Number: 12/11/1/18

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -07- 01

SUBJECT: Molecular epidemiology of bovine fasciolosis in smallholder cattle farming areas of North West Province, South Africa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Humanities 100
1919 - 2019
Research Ethics Committee

14 October 2019

Dear Dr MC Marufu

Project Title:	Assessment of the level of knowledge, attitudes and practices on bovine fasciolosis of smallholder cattle farmers in North West Province, South Africa
Researcher:	Dr MC Marufu
Supervisor:	
Department:	External department
Reference number:	04915365 (REC086-19)
Degree:	Doctoral

I have pleasure in informing you that the above application was **approved** by the Research Ethics Committee on 27 June 2019. Data collection may therefore commence.

Please note that this approval is based on the assumption that the research will be carried out along the lines laid out in the proposal. Should the actual research depart significantly from the proposed research, it will be necessary to apply for a new research approval and ethical clearance.

We wish you success with the project.

Sincerely

Prof Maxi Schoeman
Deputy Dean: Postgraduate and Research Ethics
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: PGHumanities@up.ac.za

Appendix 6: Acceptance Certificate from *Pathogens*

pathogens

an Open Access Journal by MDPI

Indexed in: PubMed | CITESCORE 3.5 | IMPACT FACTOR 4.531

CERTIFICATE OF ACCEPTANCE

Certificate of acceptance for the manuscript (**pathogens-1999289**) titled:

Comparison of three diagnostic methods to detect the occurrence of Fasciola species in communally grazed cattle in the North West Province, South Africa

Authored by:

Sunday C. Olaogun; Charles Byaruhanga; Sunday O. Ochai; Geoffrey T. Fosgate; Munyaradzi C. Marufu

has been accepted in *Pathogens* (ISSN 2076-0817) on 20 November 2022

MDPI Academic Open Access Publishing

Basel, November 2022

Appendix 7 Acceptance letter from Tropical Animal Health and Production Journal

SPRINGER NATURE

Dear Dr. Munyaradzi Christopher Marufu

We're delighted that your article has been accepted for publication: 'The knowledge, attitudes, and practices of smallholder cattle farmers concerning the epidemiology of bovine fasciolosis in the North West Province, South Africa'.

You now need to:

- Provide details to help us check whether your article processing charge (APC) is covered by your institution or a journal partner.
- Read and accept your publishing agreement.
- Make a payment if the APC is not covered.

If you want to publish subscription, instead of open access, there will be an option to do that in the following steps.

Continue with publishing your article:

https://publishing-and-rights.springernature.com/workflow/b4407bfb-c0f0-4177-b6fb-ae9db6f45016

Kind regards,

Springer Nature Author Service

If you have any questions, please do not hesitate to contact our Author Service team at ASJournals@springernature.com