DEVELOPMENT OF MOLECULAR DIAGNOSTIC METHODS (LAMP AND PCR) FOR DETECTION OF *HAEMONCHUS CONTORTUS*, *FASCIOLA* SPP AND *TRICHOSTRONGYLUS* SPP INFECTIONS IN LIVESTOCK

By

Lerato Tshepiso Mabe

Dissertation submitted in fulfilment of the requirements for the degree Magister Scientiae in the Faculty of Natural and Agricultural Sciences, Department of Zoology and Entomology, University of the Free State

Supervisors: Dr. A.M. Tsotetsi & Dr. O.M.M. Thekisoe

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SUPERVISORS

Dr. Ana M. Tsotetsi

Parasites, Vectors and Vector-borne Diseases Programme

ARC-Onderstepoort Veterinary Institute

Private Bag X05

Onderstepoort

0110

Dr. Oriel M.M. Thekisoe

Parasitology Research Program

Department of Zoology and Entomology

University of the Free State Qwaqwa Campus

Private Bag X13

Phuthaditjhaba

9866

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that it has not, previously in its entirety or in part, been submitted at any university for a degree. I therefore cede copyright of this dissertation in favour of the University of the Free State.

Signature :.....

Date :.....

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ABBREVIATIONS

ARC-OVI	Agricultural Research Council-Onderstepoort Veterinary Institute
BSA	Bovine serum albumin
COX1	Cytochrome C Oxidase subunit 1
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPG	Egg per gram
FD	Fluorescent reagent
ITS2	Internal transcribed spacer 2
L_3	Infective third stage larvae
LAMP	Loop-mediated isothermal amplification
NaCl	Sodium chloride
NaOAc	Sodium acetate
NZG	National Zoological Gardens
PBS	Phosphate buffered saline
PBS	Phosphate buffer saline
PCI	Phenol chloroform isoamyl alcohol
PCR	Polymerase chain reaction
PVC	Packed cell volume
qPCR	Real-time PCR (Quantitative PCR)
Tris-HCl	Tris-Hydrochloric acid
UV light	Ultra violet light

ABSTRACT

Helminths belonging to the genera *Haemonchus* and *Trichostrongylus* as well as those of the genus *Fasciola* are the causative agents of various helminthiases in animals and sometimes in humans. In both host types infections are often acquired through the ingestion of infected food as well as drinking contaminated water. In animals they disrupt the efficient conversion of food material and absorption of nutrients resulting in weakness and death. Infected humans often suffer from intestinal obstruction, insomnia, vomiting, weakness and stomach pains and sometimes temporary asthma. Infections are associated with huge economic losses globally. Diagnosed is achieved through the observation of clinical manifestations. In low infections alternative diagnosis is based on the recovery and identification of faecal eggs and cultivation of L_3 by microscopy. More accurate diagnosis has recently been achieved through the use of molecular diagnostic tools such as PCR and LAMP.

The aim of this study was to develop rapid, sensitive, specific and accurate molecular diagnostic assays. In particular the study focused on LAMP and PCR for the detection of *H. contortus*, *Fasciola* and *Trichostrongylus* spp. infections in four provinces of South Africa.

The first study emphasized on the development of LAMP and PCR assays for detection of *H. contortus* infections in livestock. LAMP primers that specifically amplify the ITS2 gene of *H. contortus* were designed from this target gene. This set of primers was used to develop a PCR assay for species-specific gene amplification. Both assays were tested at various reaction conditions to optimize for primer annealing temperature. Sensitivity reactions were conducted using 10 fold serial dilutions of target DNA while primer specificity was determined using DNA extracted from closely related species. For the LAMP assay, the optimum annealing temperature was found to be 60°C and 55°C for the PCR assay. When tested for specificity both assays only amplified target DNA thereby proving to be specific. The sensitivity reactions for both the LAMP and PCR assay yielded a detection limit of 0.42 ng and 10^{-3} ng respectively as the lowest amount of the target DNA that can be detected by the assays. Screening of field samples by PCR yielded negative results on several occasions while the positive control amplified the target gene as

expected. Failure to validate the assay using field samples was attributed to poor quality or lack of DNA. Validation of these assays is central to determining their efficacy and potential importance in diagnosing natural infections with high sensitivity and rapidity. Therefore we recommend close evaluation of DNA extraction from faecal material as well as ways of reducing or eliminating PCR inhibitors.

The second study was aimed at developing a LAMP and PCR assay for the detection of *Trichostrongylus* spp. infections in livestock. In this study, the target gene used for LAMP primer design for genus-specific amplification was the ITS2 gene. Two primer sets were designed and from these primers, two PCR assays were developed. All these assays were subjected to various LAMP and PCR conditions respectively in order to determine suitable annealing temperatures for each assay. Various methods of DNA extraction were evaluated for troubleshooting together with the use of already published PCR primers. Both LAMP and PCR assays did not amplify the target gene at different ranges of annealing temperatures tested. Furthermore, no amplification was achieved from control DNA samples extracted using different methods. Negative results were obtained when the PCR was troubleshot using already published primers. The results achieved with the designed assays may be a direct consequence of improperly designed primers; however, the inability of all primer sets to function for LAMP and PCR as well as for already published primers suggests problems with the extraction of DNA. This can be attributed to ineffective disruption of worms or the presence of DNases that may degrade DNA and inhibit its amplification. The results of this study suggest the need to evaluate pre-treatment of worms prior to DNA extraction. Evaluation of the methods used to reduce the effects of PCR inhibitors during extraction and amplification of DNA may also be considered.

The third study was aimed at developing a LAMP and PCR assay for detection of *Fasciola* spp. infections in livestock. Two sets of primers for species-specific amplification by LAMP were designed by targeting the ITS2 gene of both *F. hepatica* and *F. gigantica*. Species-specific PCR assays were developed from the latter primers and gene of the parasites and the assays were then tested at various reaction conditions. A genus-specific PCR assay was subsequently developed and tested. No amplification of DNA was observed with the *F.hepatica*-specific LAMP assay whereas the assay developed for specific detection of *F. gigantica* produced

false positive results. All PCR assays yielded negative results following many attempts to optimize for primer annealing temperature. Reagent contamination was eliminated as the source of non-specific amplification with LAMP suggesting that improper primer design was a possible cause of this problem. On the other hand failed attempts to optimize all the other assays suggest that there is need to evaluate pre-treatment of worms prior to DNA extraction as well as the methods reducing the effects of PCR inhibitors during amplification.

Overall, this study successfully achieved the development and optimization of a LAMP and PCR assay for detection of *H. contortus* DNA. However, subsequent validation of the assays using field derived samples was not possible. The overall results of this study mostly point to faulty primer design and the presence of PCR inhibitors in extracted DNA samples, extracted from either tissue or faecal samples. According to previous studies the presence of inhibitory substances may interfere with the lysis step, inactivate the thermostable DNA polymerase and even interfere with nucleic acids. Therefore evaluation of more accurate methods of mechanical disruption of the worms, DNA extraction, primer design and the use of amplification facilitators may yield desired results. Therefore, these factors should be taken into account for successful development and validation of the molecular diagnostic tool for detection of helminth infections.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Helminth is a general term that applies to the parasitic worms. Helminths are parasitic because they survive on the nourishment they receive from their hosts as well as the protection they need to survive, all of which happens at the expense of the host (Hale, 2006). Helminths include the monogeneans, which includes the nematodes (roundworms), trematodes (liver flukes) and the cestodes (tapeworms). These parasites often infect animals and/or humans through vector bites, ingestion of contaminated food and water and walking barefoot on infected soil. Helminth infestations often disrupt the ability to efficiently convert food material and absorb nutrients, the result of which is weakening and even death. This poses a serious threat on human health and on the production of animals which in turn impacts negatively on the economy (Gasser *et al*., 2008; Bott *et al*., 2009; Ahmadi and Meshkehkar, 2010; Ahmed, 2010; Sweeny *et al*., 2011), as there is reduced yield and quality of products such as meat, milk and wool (Schnieder *et al*., 1999; Tsotetsi and Mbati, 2003; Aldemir, 2006; Maphosa *et al*., 2010), not forgetting the condemnation of infected meat at abattoirs (Cucher *et al*., 2006).

Helminth parasites may be found either attached to the intestinal wall of their host where they feed on blood causing anemia or on other parts of the body where they consume nutrients causing malnutrition and weight loss (Hale, 2006). Briefly, the effects of the infections include diminished food intake, reduced food conversion efficiency, weight loss, reduced fertility, reduced energy to carry out day to day activities and death (Perry and Randolph, 1999; Ahmed, 2010). Infected human beings often suffer intestinal obstruction, insomnia, vomiting, weakness and stomach pains and sometimes temporary asthma (Lattès *et al*., 2011). The burden of infections is more evident in developing countries as these regions seldom have a clean supply of water and food as well as the luxury to afford shoes and clothing. Again because of the high costs that are associated with the treatment of parasitic infections, the impact is more apparent in developing countries due to the lack of veterinary care and finances (Ahmed, 2010). Temperature, rainfall and humidity all

influence the development and growth of eggs and larvae of most nematodes. Generally, parasites prefer and thrive in climates such as those found in the tropics and subtropics (Hale, 2006; Ahmed, 2010). Unfortunately that is where most developing countries lie and for this reason the developing world finds itself more susceptible to infection than does the developed world (Ahmed, 2010). Due to the long existence of parasite-host relationships, there is some level of resistance to infection by livestock but the effects of infection still outweigh resistance mechanisms by hosts as infected animals exhibit signs of low productivity performance as measured by indicators such as weight gain, age at first calving and calving percentages (Perry and Randolph, 1999). The use of drug treatment to control parasitic helminths requires rapid and accurate diagnostic methods for successful treatment of the parasitic infestations. There is therefore urgency in development of rapid and accurate diagnostic tools for successful implementation of effective strategic management plans and monitoring anthelminthic drug efficacy (Gasser *et al*., 2008; Sweeny *et al*., 2011).

1.2 General life cycle of helminths

The species that form part of the larger group of parasitic worms collectively known as helminths are vastly different, with life cycles that vary from simple to those that are a bit more complex. Although this is the case there is some level of similarity between the life cycles of most if not all of these parasitic worms. Firstly, all parasitic helminths undergo three main developmental stages in their life cycle, which is development from eggs to larvae to adults (**Fig 1a**). Secondly, all adult worms are found in the definitive host while larval stages may be either free-living in water or soil or may be found in intermediate hosts. One other feature that is common amongst most helminths (with the exception of others, which will be detailed later on in this section) is the production of eggs that are shed in the faeces. These eggs hatch and develop into infective larval stages at optimal environmental conditions (temperature, rainfall and humidity). From here on the larvae will either actively infect intermediate hosts and undergo further development (**Fig 1b**) after-which they will be released into the water and actively infect the definitive host or encyst on vegetation, which will become infective upon consumption. In most of species the infective stage of the life cycle is the L_3 larvae, however, with other species like the Ascarids and pinwowms it is the L_1 larval stage that appears to be infective. As mentioned earlier

on, not all helminths produce worms that are shed in the faeces. Some are viviparous infecting intermediate hosts through arthropod bites, in some the L_1 larvae are found in the faeces and still with others the L_1 develop into L_3 within the definitive host (animal host) and encyst on the muscles. In such cases as the latter mentioned the definitive host (human host) gets infected through the consumption of raw or undercooked contaminated meat (Jimenez-Cisneros and Maya-Rendon, 2007). The immense diversity found in helminths is illustrated in **Fig 1c**, **d** and **e** below.

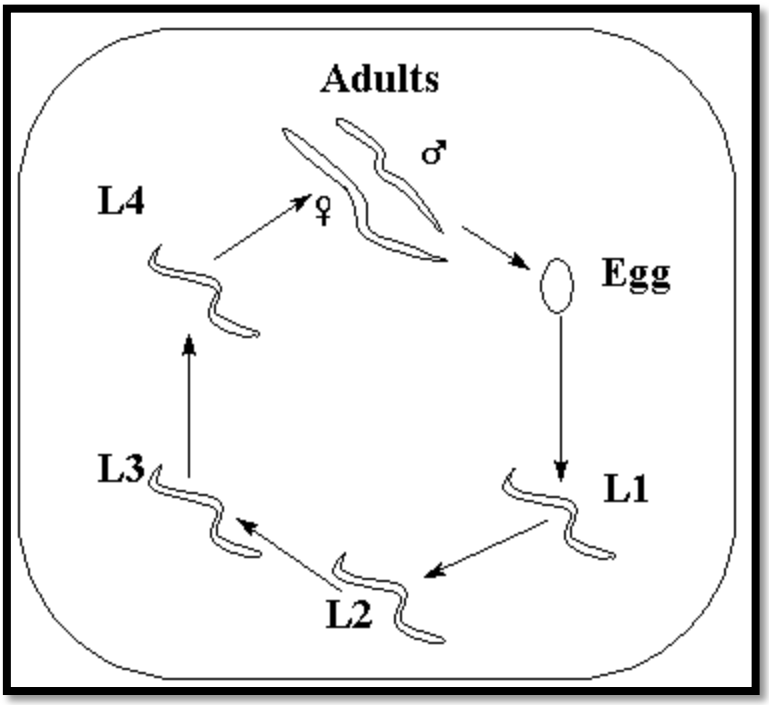


Fig 1a: Basic representative life cycle of helminths. (L1) First larval stage, (L2) Second larval stage, (L3) Third larval stage, (L4) Forth larval stage. Figure extracted from http://www.personal.psu.edu/ncj111/Unique%20Features.htm

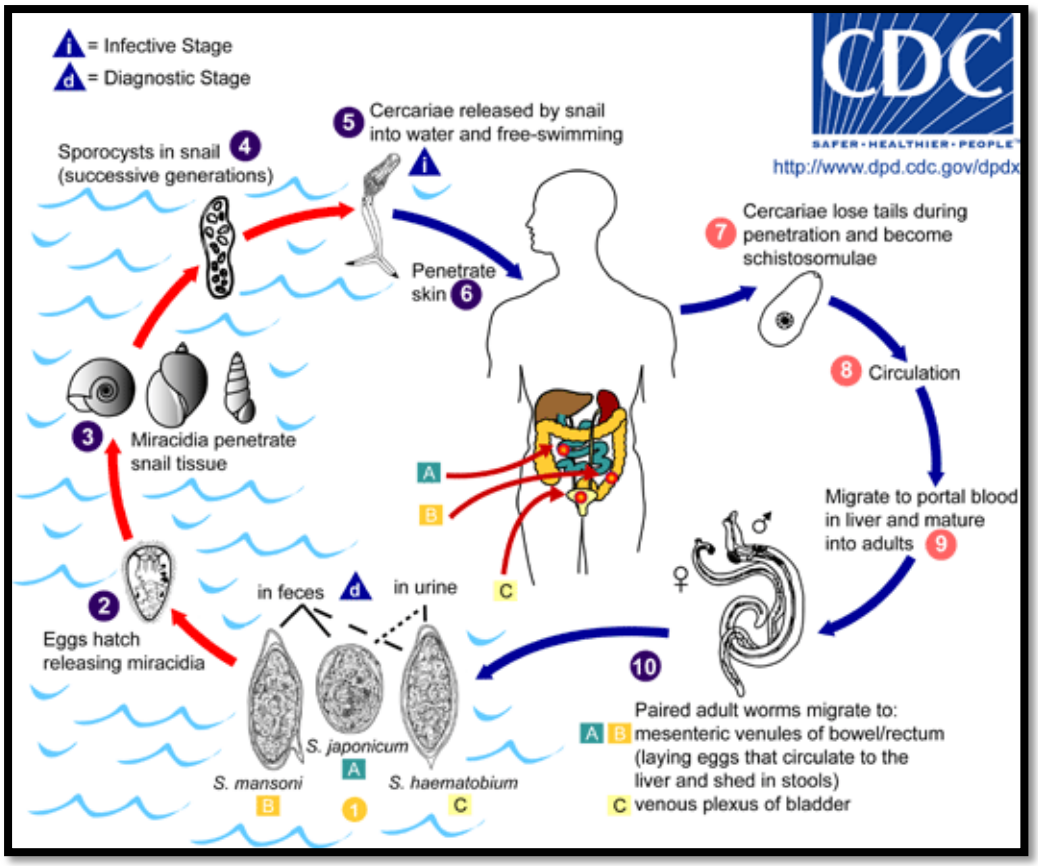


Fig 1b: Life cycle of *Schistosoma* spp. A typical example of a complex life cycle where an intermediate host is required in order to complete the life cycle. Figure extracted from http://www.cdc.gov/parasites/schistosomiasis/biology.html

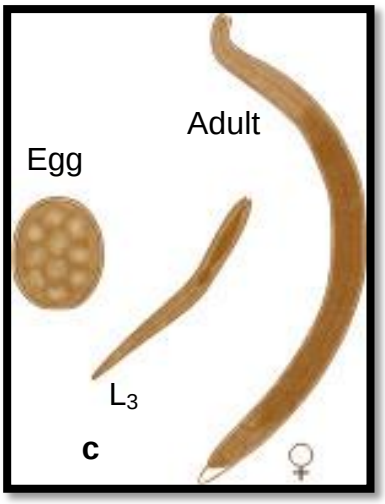


Fig 1c: Developmental stages of nematodes. Figure extracted from http://parasite.org.au/para-site/contents/helminth-

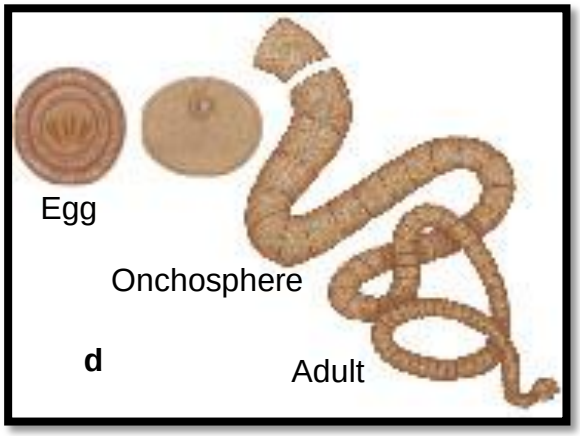


Fig 1d: Developmental stages of cestodes. Figure extracted from http://parasite.org.au/para-site/contents/helminth-intoduction.html

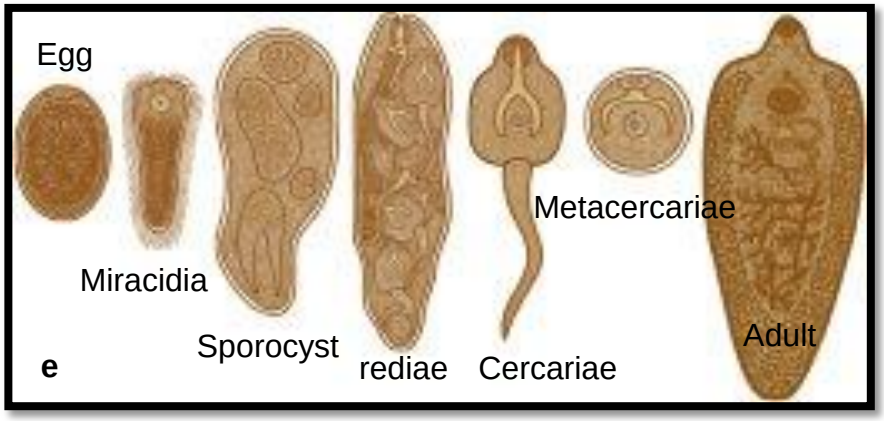


Fig 1e: Developmental stages of trematodes. Figure extracted from http://parasite.org.au/para-site/contents/helminth-intoduction.html

1.3 Economic and human health threat

According to research , economic losses due to infection with gastrointestinal nematodes amount to millions or even billions of rands annually in many countries . The cost of infection in Australia is estimated at ~1 billion Australian dollars annually (Gasser *et al*., 2008; Bott *et al*., 2009), while in America the cost of infection is estimated at 2 billion US dollars per annum to the cattle industry (Zarlenga *et al*., 2001). A greater percentage of the loss of productivity in small ruminant industries in developing countries is attributed to infection with gastrointestinal nematode like *Haemonchus contortus* (Hounzangbe-Adote *et al*., 2005). In addition, the costs that are associated with strategic control of nematodes using anthelmintics restrict effective control and management of gastrointestinal parasites in developing countries (Hounzangbe-Adote *et al*., 2005; Hale, 2006; Bott *et al*., 2009). Weight loss which results in less production of meat together with subsequent deaths following heavy infection with internal parasites contributes greatly to economic losses (Hale, 2006). Several studies have documented an increase in human population infections by members of the genus *Fasciola*. Human infections are documented to have reached a high of 2.4 -17 million people and higher according to recent studies (McGarry *et al*., 2007; Ahmadi and Meshkehkar, 2010; Duthaler *et al*., 2010) and a further 90-180 million people estimated to be at risk of infection with countries such as Bolivia, Peru, Egypt, the eastern Mediterranean, Vietnam and China being at the top of the list (Alasaad *et al*., 2011). More than 250 and 300 million sheep and cattle respectively, are reported to be affected by fascioliasis resulting in an economic loss of more than R20 billion to the agricultural sector each year (Duthaler *et al*., 2010).

Nematodes of the genus *Trichostrongylus* have also been implicated as major contributors to economic losses due to their impact on animal health (Hoste *et al*., 1995). Studies have also documented increasing cases of *Trichostrongylus* infection in humans (Wall *et al*., 2011). A substantial amount of these infections is believed to have been underestimated and even overlooked due to their rarity and mistaken identity as hookworm infections.

According to studies on management of gastrointestinal nematodes *H. contortus* is a huge concern in the small ruminant industry (Tsotetsi and Mbati, 2003; Hale, 2006). Infections are particularly difficult to manage because of the increased resistance

that the parasites have developed against commercial drugs (Hounzangbe-Adote *et al*., 2005; Hale, 2006; Tiwari *et al*., 2006). Resistance is the ability of the worms in a population to survive doses of drug treatment that would normally get rid of them at a certain stages of infection (Prichard, 2001; Hale, 2006). In order to understand resistance we need to understand the factors that play a role and contribute to the development and reinforcement of resistance. Resistance develops when unsustainable methods of de-worming are used, be it due to lack of knowledge or for any other reason (Prichard, 2001; Hale, 2006). These methods include de-worming for periods longer than is prescribed, administration of lower doses than is required due to underestimations of body weight, and treating even uninfected animals. Other de-worming methods that may contribute to drug resistance include treatment of animals and then moving them to infection free pasture where they will deposit resistant strains of the parasite in the form of eggs in the faeces (Hale, 2006).

Anthelminthic treatment eliminates parasites that are susceptible to the drugs but those whose genotype renders them resistant will survive the treatment and therefore be able to pass on the resistant genes to future generations of worms (Prichard, 2001; Hale, 2006). According to Prichard (2001), “The frequency and intensity of treatment, and the extent of dilution of ‘resistance’ alleles by ‘susceptibility’ alleles in the reproducing population, by parasites that establish after the treatment, determine the rate of selection for resistance.” Therefore, continued treatment allows for increased resistance as there will be less ‘susceptible’ genes in the population to dilute the ‘resistance’ genes (Prichard, 2001). Due to the excessive use of anthelminthics, worms (with emphasis on *H. contortus*) have built up resistance to almost all commercially available drugs (Hounzangbe-Adote *et al*., 2005; Hale, 2006; Tiwari *et al*., 2006). The faecal egg count reduction test, the egg hatch assay and larval development assay are three of the most widely used assays for detection of anthelminthic resistance. However, they are limited in sensitivity, require a lot of strenuous work and are costly (Tiwari *et al*., 2006). Therefore, because resistance has become a worldwide phenomena (Hale, 2006), there is an increased need for the development of rapid, sensitive and specific diagnostic assays for better evaluation of drug treatment and to improve herd production and management (Harmon *et al*., 2007).

1.4 Diagnosis of helminth infections

Diagnosis of helminth infections is primarily based on the observation of clinical symptoms wherever possible. However, this method of diagnosis is often unreliable due to the absence of symptoms in many cases, more so at low infection intensities. One other disadvantage of relying on clinical symptoms for diagnosis is that in mixed infections, it becomes near impossible to point out the causative agent(s) (Lattès *et al*., 2011). Due to difficulties encountered with the absence of symptoms, biologists have turned to the use of microscopic techniques to detect infections. Diagnosis in this case relies on the recovery of parasite eggs in the faeces. The eggs can be recovered by using the either sedimentation or floatation techniques, such as Visser sieve technique (Maphosa *et al*., 2010) which has both sedimentation and floatation phases/stages and a McMaster technique (Reinecke, 1983), which is basically a flotation technique. On recovery, the eggs are identified, counted and the egg per gram (EPG) of faeces is calculated to determine an estimate of the level of infection. Species whose eggs exhibit similar morphological characters and therefore cannot be distinguished from each other can further be subjected to larval culture and identification of the third stage larvae (L_3) (von Samson-Himmelstjerna *et al*., 2002). In some species like *Strongyloides stercoralis*, the eggs hatch while still inside the body of the definitive host and therefore the L_1 larvae are shed with the faeces, these can then be recovered and identified. When calculating the EPG of faeces it is important to note the unreliability of the outcome as this does not necessarily correlate with the number of adults that are present and reproducing in the gastrointestinal tract (Lattès *et al*., 2011). The use of microscopy as a diagnostic tool is not only unreliable but is also time consuming, labour intensive and limited in sensitivity and specificity.

In search for better diagnostic tools serological and immunological assays have also been developed and tested for accurate and rapid diagnosis of helminths but these were also found to be limited in specificity due to their cross reactivity (Lattès *et al*., 2011). Another disadvantage associated with antigen use is the inability to distinguish between past and present infections (Lattès *et al*., 2011). The focus has thus shifted towards the development of molecular tools for rapid and accurate detection of helminth infections. These are not free from limitations as they also cannot distinguish between patent and non-patent infection (Yong *et al*., 2007).

However, they still prove more accurate, sensitive and specific when compared to latter methods of diagnosis.

These molecular tools include the polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). The PCR was first developed in the 1980's by Kary B. Mullis (Kaltenboeck and Wang, 2005; Lattès *et al*., 2011). It is a quick and easy method of generating millions of copies of DNA from a tiny source material, even if the material is old and of poor quality. During amplification the DNA polymerase enzyme is used to copy genetic material and produce copies of the material from any DNA source (i.e. blood, hair, or tissue specimens, microbes, animals, or plants), some of which are thousands or possibly even millions of years old. Thanks to the sensitivity and specificity of the technique scientists are now able to detect microorganisms that were otherwise difficult to culture or to confirm their presence by analysis of their products (Kaltenboeck and Wang, 2005).

Conventional PCR has become popular as the diagnostic tool of choice when it comes to detecting agents of infection. This is justified by its sensitivity and specificity, more so when it is used in conjunction with probe-based DNA technologies (Weiss, 1995). Although it has been the preferred method of diagnosis thus far, faecal material contains inhibitors that potentially have adverse effects on the sensitivity of conventional PCR assays (Harmon *et al*., 2007). Another drawback of the PCR is that the risk of post-amplification contamination is high. However, other PCR methods have been developed to improve the efficiencies and sensitivities of the methods. It has been documented that the effects of PCR inhibitors in faecal samples can be reduced through the use of bovine serum albumin (BSA) during amplification and phytase during DNA extraction (Harmon *et al*., 2007).

LAMP developed by Notomi *et al.* (2000), is a novel DNA amplification technique that has the ability to amplify a single copy of DNA into a billion copies under isothermal conditions (Mori *et al*., 2001; Savan *et al*., 2005; Thekisoe *et al*., 2007). According to Ai *et al*. (2010a), the assay is "based on the principle of strand displacement and reaction and stem loop structure that amplifies target with high degree of specificity, selectivity and rapidity under isothermal conditions therefore obviating the need for use of a thermal cycler". The assay owes its sensitivity, specificity and rapidity to the use of four to six specially designed primers that recognize and anneal to six to eight

regions respectively on target DNA (Mori *et al*., 2001; Savan *et al*., 2004; Savan *et al*., 2005; Thekisoe *et al*., 2007). The reaction can be run using a common laboratory heat block or a temperature regulated water bath in addition to a thermal cycler. Results can be obtained in as early as 30 - 60 min (Mori *et al*., 2001; Thekisoe *et al*., 2007). With LAMP not only is there a large number of product DNA produced but also a proportional amount of the by-product magnesium pyrophosphate which results in the formation of turbidity in the reaction tube (Mori *et al*., 2001). This feature of LAMP makes it possible to quantify the amount of DNA produced with the use of a simple photometer or a LAMP turbidimeter. The turbidity also makes detection with the naked eye possible under normal light without any post-amplification manipulation of the amplicon such as running on an agarose gel electrophoresis. Positive amplification is indicated by turbidity in the reaction tube (Mori *et al*., 2001) while negative samples remain clear or colourless.

The addition of a fluorescent detection reagent/dye to the reaction mixture before amplification results in a colour change in the contents of the reaction tube, that is from orange (colour of the fluorescent detection reagent) to a greenish colour in positively amplified samples. These methods of detection require no post-amplification handling of amplicon and therefore significantly reduce the risk of product carryover and post-amplification contamination. Other than this, an intercalating dye can be added to the reaction product after amplification and the tubes may then be viewed under UV light (Thekisoe *et al*., 2009). In this case positively amplified samples tend to fluoresce and the opposite is true for no amplification. As with other amplification methods agarose gel electrophoresis is another possible method of analysing LAMP results. A study conducted by Thekisoe *et al*. (2009) demonstrated the stability of LAMP reagents in comparison to those of the traditional PCR. In their work they showed how LAMP reagents kept at room temperature still maintained their efficacy, an added advantage that gives the assay great potential to be applied in resource poor facilities around the globe (Thekisoe *et al*., 2009).

In South Africa, much like the rest of the world there is currently very little work done with regard to development of molecular diagnostic tools for detection of helminth infections.

1.5 OBJECTIVES OF THE STUDY

1.5.1 Statement of the problem

On a global scale the infection of ruminants with parasitic worms is a major cause of production loss (Schnieder *et al*., 1999). This is measured in terms of weight loss, reduced quantity of meat, milk and wool (Tsotetsi and Mbati, 2003; Aldemir, 2006; Maphosa *et al*., 2010). According to Schnieder *et al*., (1999) in order to device the best possible management strategies it is best we be able to differentiate between the genera of gastrointestinal nematodes as, “the epidemiology and susceptibility of these worms to drug treatment varies between genera”. Due to the cunning similarities of eggs of most of these genera microscopic identification or differentiation to species level is not possible (Bott *et al*., 2009).

Considering the difficulty to differentiate parasite eggs for species identification biologists rely on larval culture for identification of third stage (L_3) larvae (Schnieder *et al*., 1999; Harmon *et al*., 2007; Bott *et al*., 2009). However, there are several disadvantages of using larval culture techniques. For one, the procedure is time consuming with a waiting period of 5 - 8 days to complete (Schnieder *et al*., 1999; Bott *et al*., 2009; Sweeny *et al*., 2011). Secondly, it requires extensive knowledge of the subject(s) in question (Harmon *et al*., 2007; Sweeny *et al*., 2011) and the procedure is not always reliable due to subtle morphological differences that may be missed during identification (Schnieder *et al*., 1999; Bott *et al*., 2009). Reliability of microscopic techniques to give accurate indications of infection intensities is questionable as it depends on the stage of infection and the recovery of eggs in the faeces, which is illustrated by Andrews (1939). Microscopic techniques therefore in general lack the sensitivity to detect low infection intensities, the specificity to make species delineations and are laborious, time consuming and often inaccurate, hence there is a need for molecular techniques not to replace microscopy but to supplement existing methods of diagnosis.

1.5.2 General Objective

To develop molecular diagnostic assays (LAMP and PCR) for detection of *Haemonchus contortus*, *Fasciola* spp and *Trichostrongylus* spp infections in livestock.

1.5.3 Specific Objectives

- To develop a LAMP assay for detection of *Haemonchus contortus* infections in cattle, sheep and goats.
- To develop a LAMP assay for detection of *Fasciola* species infections in cattle, sheep and goats.
- To develop a LAMP assay for detection of *Trichostrongylus* species infections in cattle, sheep and goats.
- To develop a PCR assay for detection of *Haemonchus contortus* infections in cattle, sheep and goats.
- To develop a PCR assay for detection of *Fasciola* species infections in cattle, sheep and goats.
- To develop a PCR assay for detection of *Trichostrongylus* species infections in cattle, sheep and goats.
- To apply the newly developed LAMP and PCR assays in determining the prevalence of respective parasites in several provinces of South Africa.

CHAPTER 2

DEVELOPMENT OF LAMP AND PCR ASSAYS FOR DETECTION OF *HAEMONCHUS CONTORTUS*

2.1 INTRODUCTION

2.1.1 Introduction and classification of *Haemonchus contortus*

Kingdom: Animalia
Phylum: Nematoda
Class: Secernentea
Order: Strongylida
Family: Trichostrongylidae
Genus: *Haemonchus*
Species: *Haemonchus contortus*

Haemonchus contortus is a cylindrical gastrointestinal nematode commonly known as the red stomach worm, the wire worm or the Barber pole worm (Hale, 2006). It is a cosmopolitan species but prefers and therefore occurs in large numbers in subtropical and tropical regions of the world (Waller and Chandrawathani, 2005). Adult worms are found in the abomasum of goats and sheep (Schallig *et al,* 1995; Tiwari *et al*., 2006; Inaam *et al*., 2007). According to Maphosa *et al*., (2010) *H. contortus* is a highly pathogenic parasite of small ruminants, capable of causing acute disease and high mortality in all classes of stock. These parasites produce large numbers of eggs per parasite per day; this together with suitable climates of high temperature and rainfall ensures year-round, undisrupted development of larvae (Maphosa *et al*., 2010). *H. contortus* infection is so significantly high and of importance that it made the top ten list of the most common pathogenic nematodes of sheep and goats in the tropics (Maphosa *et al*., 2010), and together with other nematodes, the second most common gastrointestinal infection that results in death of sheep in Malaysia (Cheah and Rajamanickam, 1997). According to Waller and Chandrawathani (2005), gastrointestinal nematode infection ranks highest on a global index, with *H. contortus* being of overwhelming importance.

2.1.2 Life cycle of *H. contortus*

Eggs are passed out in the faeces of the mammalian host. The eggs hatch and develop into the L_1 and L_2 juvenile stages in the faeces while feeding on bacteria. The L_1 stage usually takes about 4 - 6 days to develop under temperatures in the range of 24 - 29°C. The L_2 stage then develops into the L_3 stage, which is referred to as the filiariform infective larvae, by shedding its cuticle. The L_3 stage remains in its cuticle and crawls up the grass blades awaiting ingestion by a final host (the herbivore), which becomes infected post-ingestion. This larva settles in the abomasum where it sheds its cuticle and burrows into the abomasum layer where it develops into the pre-adult stage (L_4). This larva in turn sheds its cuticle and develops into the adult stage (L_5). Male and female worms mate and live in the abomasum where they feed on the blood of the host (**Fig 2**). The life cycle reported here is extracted from Bowman, (2003) and Hale, (2006).

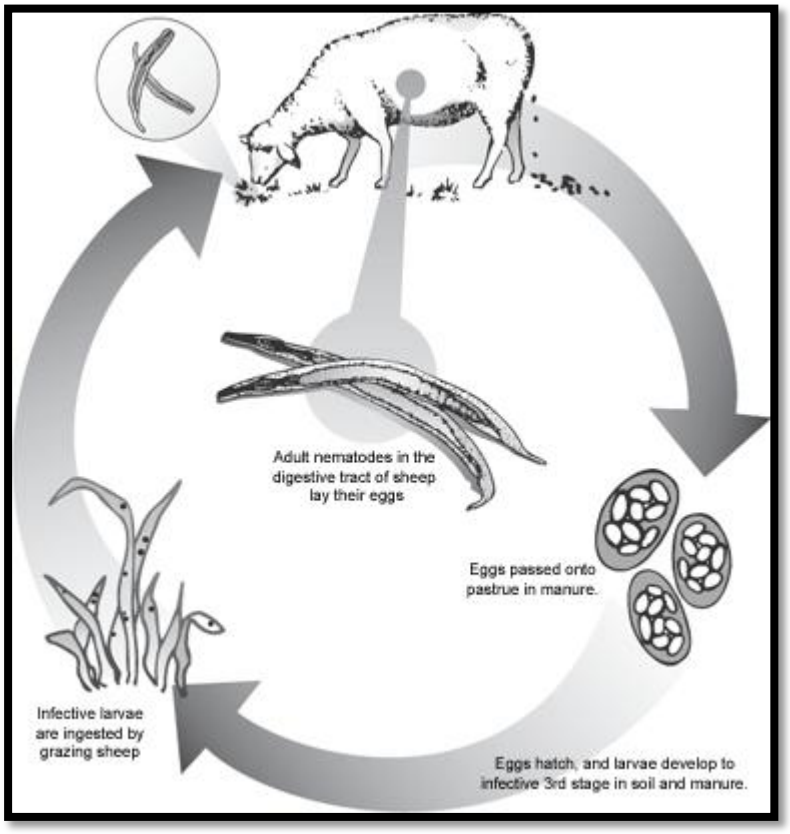


Fig 2: Life cycle of *Haemonchus contortus*. Figure extracted from http://pubs.ext.vt.edu/410/410-027/410-027.html

2.1.3 Diagnosis of *H. contortus* infections

Haemonchus contortus infection is clinically diagnosed by anemia, dehydration, sub-mandibular internal fluid accumulation that results in the formation of a bottle neck, diarrhoea and low packed-cell volume (PCV), (Inaam *et al*., 2007). Infection also results in retarded growth, reduced reproductive performance, general illness and death, (Schallig *et al,* 1995).

Supplementary diagnosis is achieved through the use of microscopic techniques by the recovery of *H. contortus* eggs from stool samples (Schallig *et al,* 1995). Because the eggs of many important genera are morphologically similar and therefore hard to identify to species level, a better way to delineate species is by larval culture and identification of 3rd stage larvae (Schnieder *et al*., 1999; Harmon *et al*., 2007).

Alternative diagnosis is based on serological techniques including the likes of the ELISA (Schallig *et al*., 1995) and dot-ELISA (Prasad *et al*., 2008). According to Thekisoe *et al*., (2007) the biggest drawback of tests that rely on the detection of antibodies is their inability to distinguish between past and present infections. Molecular techniques have proved more rapid and accurate than both microscopy and serology hence there has been recent movement towards the development of molecular assays for detection of helminth infections.

A qPCR for detecting trichostrongyle infections, including *H. contortus*, in ruminants was developed by Von Samson-Himmelstjerna *et al*., (2002). The assay consisted of four genus-specific primer/probe sets enabling sensitive and specific amplification of target DNA from four trichostrongyle genera in the presence of multiple infections. Many other successful PCR-based assays have been developed over the past decade for identification and differentiation of strongyle infections (Schnieder *et al*., 1999; Zarlenga *et al*., 2001; Von Samson-Himmelstjerna *et al*., 2002; Lier *et al*., 2006; Learmount *et al*., 2009; Alasaad *et al*., 2011). However, qPCR assays are more expensive as they include the use of expensive probes hence alternative and cheaper methods of diagnosis are a necessity, justifying the need to develop a PCR assay for detection of *H. contortus* infections as a supplementary diagnostic tool.

A number of LAMP-based assays have been designed for detection of parasitic infections, unfortunately none have so far been developed for identification of *H.*

contortus infections. Hence, the current study endeavours to develop a LAMP assay, which is specific for diagnosis of *H. contortus* infections.

2.1.4 Objectives

- To develop a LAMP assay for detection of *H. contortus* infections in cattle and sheep.
- To develop a PCR assay for detection of *H. contortus* infections in cattle and sheep.
- To apply the newly developed LAMP and PCR assays in prevalence studies of *H. contortus*

2.2 MATERIALS AND METHODS

2.2.1 Helminth samples used for positive and negative control purposes

For the purpose of this study adult worms (*Haemonchus placei*, *Fasciola hepatica*, *F. gigantica*, *Trichuris spp.*, *Oesophagostomum radiatum*, *Trichostrongylus* spp., *Schistosoma mattheei*, *Cooperia* spp., *Avitellina* spp., and *Paramphystomum* spp), and third stage larvae (L_3) of *H. contortus* cultured from faecal eggs and identified in the laboratory according to van Wyk *et al*., (2004) were obtained from the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, South Africa. The larvae were preserved in ddH_2O and kept at 4°C whereas, all adult worms were preserved in formalin at room temperature. Some of the worms were transferred to 1.5 µl eppendorf tubes containing 75% ethanol for long term storage and then kept at -34°C until used for DNA extraction.

2.2.2 Faecal sample collection and storage

Fresh faecal samples were collected from animals raised in four South African Provinces including the Free State, Gauteng, Mpumalanga and Kwa-Zulu Natal. A total of 266 samples were collected from sheep, cattle and goats. A detailed account of the number of samples collected in each province is presented in **Table 3**. The samples were collected directly from the recta of these ruminants in the field and then placed in separate plastic bags that were labelled accordingly and kept in a cooler bag with ice packs. The samples were later stored at 4°C and the air was expelled from the bags to allow later examination. For optimization purposes examination by microscopy was conducted within 24 hours of collection using the Free State samples. McMaster techniques were used for helminth diagnosis and detection while a portion of positive samples were cultured. A reasonable amount of each of the faecal samples was preserved in 1.5 µl eppendorf tubes containing PBS and stored at -34°C pending extraction of DNA at a later stage.

Table 1: Detailed account of faecal sample collection from animals in four South African provinces

Province	Cattle	Sheep	Goats
Free State	13	76	ND
Kwa-Zulu Natal	33	29	52
Gauteng	41	ND	ND
Mpumalanga	ND	ND	22

***ND= Not collected

2.2.3 Faecal Sample Processing

i. McMaster faecal egg count (Reinecke, 1983)

For sheep 2 g of faeces were weighed out and 4 g for cattle. The sheep pellets were crumbled finely and mixed with 58 ml of a 40 % salt solution. To the cattle faeces 56 ml of the 40 % salt solution was added as a floatation medium. The suspension was blended thoroughly in a blender and a few drops of amyl alcohol were added where necessary to break the foam that formed. A pasture pipette was used to mix the suspension well in order to get a random distribution of the eggs. The two chambers of the McMaster slide were immediately filled with a pasture pipette and allowed to stand for approximately 2 minutes so as to allow the eggs to float to the surface of the floatation medium. This ensured that the eggs lie in contact with the upper glass of the chamber. A light microscope was used to examine the slide and count the number of eggs in the grid of each counting chamber. Only the eggs within the walls of the grid were counted and those on the outside of the grid ignored. The number of egg per gram (EPG) of the faeces was calculated by counting all the eggs in the two chambers of the slide and multiplying them by 100 for sheep and 50 for cattle.

ii. Faecal culture for cultivation of L_3 stage (Reinecke, 1983)

Fresh faeces were measured into a container and the sheep pellets crumbled with a spatula. A similar amount of vermiculite was added to the faeces and the two were mixed together. This was to improve air circulation in the culture and to facilitate maximum hatching. A 2 cm wooden stick was placed in an upright position in the centre of a jar and the faeces added and pressed down with a second stick. The

layer of culture was 5 - 7 cm thick and the stick was removed leaving a hole in the centre, which facilitated the aeration. The exterior surface of the container was cleaned to prevent contamination. The faecal cultures were moistened by means of a water bottle, taking care not to pour too much water so as to avoid soaking the cultures. The lids were lightly screwed back on and the bottles incubated at 25-28°C for 7 days. In order to harvest the culture, the inner surface of the jars and the surface of the cultures was moistened. The cultures were then placed in the light, taking care not to expose them to direct sunlight. Finally, the larvae were collected by rinsing the sides of each jar into a separate petri-dish, allowed to sediment and then examined under a microscope. The larvae were counted and various numbers of them were then placed in 1.5 ml eppendorf tubes and stored at 4°C for future use.

2.2.4 Methods used for DNA extraction and troubleshooting

DNA was extracted from faecal samples containing nematode/helminth eggs, third stage larvae obtained from faecal cultures and adult worms by the salting out method. In order to troubleshoot two other methods of DNA extraction were tested for extraction of DNA from the adult worms and faecal samples respectively. The PCI method was further modified by addition of 1%, 1M, 0.5M and 0.05M concentrations of 2-mecarptoethanol to the extraction buffer containing the worm tissues at the digestion step. The different methods of extraction are detailed below.

i. DNA extraction by salting-out method (Aktas *et al*., 2006)

The adult worms were washed with xylene followed by two washes with 75% ethanol to remove traces of xylene. Therefore, the worms were preserved in 75% ethanol and stored at -34°C until used for DNA extraction. DNA was extracted from adult worms, larvae and faecal samples containing helminth eggs using an extraction buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate. First, 50 cm^3 of each piece of DNA source material was added into a reaction tube with a label that coincides with the sample code. To each 50 cm^3 sample, 500 µl of extraction buffer was added followed by 10 µl of proteinase K stock to the final concentration of 100 µg/ml. The samples were incubated overnight at 50 - 55°C. The next morning the samples were centrifuged for 5 min at 10 000 rpm and the supernatant transferred to another 1.5 ml collection tube. Thereafter, 180 µl of NaCl (5M) was added and mixed well by inverting sample tubes 50 times to

precipitate proteins and other cellular contents after which the contents were centrifuged again for 5 min at 10 000 rpm. The supernatant was transferred to a clean reaction tube and 500 µl of ice-cold isopropanol added to each tube and mixed by inverting the tubes 50 times to precipitate DNA. The reaction mixture was spun for 5 min at 10 000 rpm. The supernatant was discarded and each pellet was washed twice with 250 µl of 75% ethanol while spinning the tubes after each addition of ethanol and discarding the supernatant. The pellets were air dried for 1 hour at room temperature or incubated for 30 min at 37°C. The pellets were re-suspended in 150 µl of ddH_2O and the samples incubated at 37°C for 1 hr. The DNA samples were then stored in the freezer at -34°C until needed.

ii. DNA extraction by Phenol Chloroform Isoamy alcohol method (Sambrook and Russell, 2001)

DNA was extracted from adult worms, larvae and eggs using an extraction buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate. First, 50 cm^3 of each piece of DNA tissue sample was added into a reaction tube with a label that coincides with the sample code. To each 50 cm^3 of the sample, 500 µl of extraction buffer was added followed by 10 µl of proteinase K stock to the final concentration of 100 µg/ml. The samples were incubated overnight at 50 - 55°C. On the second day the reaction tubes were removed from the incubator and 550 µl of PCI was added to each tube and mixed by vortexing for 30 s and then centrifuging for 5min at 15 000 rpm. The resultant upper aqueous phase was transferred to new tubes labelled like the previous ones and another 550 µl of PCI was added, mixed vigorously and then centrifuged at full speed for 5 min. The supernatant was transferred to a new reaction tube and 550 µl of chloroform added and mixed by vortexing for 30 s. The tubes were centrifuged for 5 min at 15 000 rpm and 400 µl of the supernatant transferred to a new tube correctly labelled with the final sample code. About 40 µl of 3M NaOAc and 400 µl of Isopropanol were added to reaction tube with the final sample code and mixed by vortexing for 30 s followed by centrifugation at 15 000 rpm for 30 min. All the supernatant was removed after centrifugation and the pellet washed twice with 1 ml of 75% ethanol and centrifuged at full speed for 5 min after each wash. After the final wash the supernatant was discarded and the pellet was dried at 37°C in an open tube covered with paper towel for 15 - 30 min. The pellet was resuspended in 100 µl double distilled water and

allowed to dissolve at 37°C for 1h. The DNA sample was then kept at -34°C for future use.

iii. Preparation of Control DNA by QIAGEN QIAamp DNA mini kit – (QIAGEN, Valencia, CA, USA)

A small amount of tissue obtained from each adult worm was cut into small pieces and placed in a 1.5 ml microcentrifuge tube 180 µl of buffer ATL was added to the tissue samples. Twenty micro-litres of Proteinase K was added and the contents of the tube mixed by vortexing. This was followed by incubation at 56°C until the tissue was completely lysed. The sample was removed from the incubator occasionally for vortexing and incubated again shortly after. Following incubation the tube was briefly centrifuged to remove drops from the inside of the lid. Then 200 µl of buffer AL was added to the sample and mixed by pulse-vortexing for 15 s and incubated at 70°C for 10 min. The microcentifuge tube was briefly centrifuged to remove any drops from inside the lid. To the sample, 200 µl of absolute ethanol was added and the two were mixed by pulse-vortexing for 15 s after-which the tube was briefly centrifuged. The mixture was then carefully applied to the QIAamp Mini spin column without wetting the rim and then the column was centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and the filtrate was discarded. A volume of 500 µl of buffer AW1 was added to the QIAamp Mini column and the column centrifuged at 8000 rpm for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube and the filtrate was discarded. A volume of 500 µl of buffer AW2 was added to the QIAamp Mini column and centrifuged at 14 000 rpm for 3 min. The QIAamp spin column was fitted into a new 2 ml collection tube and the column centrifuged at full speed for 1 min. Then the QIAamp spin column was transferred into a new 1.5 microcentrifuge tube labelled with the final sample code. A volume of 200 µl buffer AE was carefully transferred directly onto the QIAamp membrane and the tube incubated at room temperature for 1 min followed by 1 min centrifugation at 8000 rpm. The elution step was repeated and the DNA kept at -34°C for future use.

iv. Extraction of DNA from faeces by QIAGEN QIAamp DNA Stool kit (QIAGEN, Valencia, CA, USA)

About 220 mg faecal sample was put in a 2 ml microcentifuge tube and then weighed. The tube containing the faecal sample was placed on ice. To the stool sample 1.4 ml of buffer ASL was added, the tube was then vortexed continuously for 1 min until the stool sample was thoroughly homogenized. The suspension was heated for 5 min at 70°C followed by vortexing for 15 s and centrifugation at full speed for 1 min at. One point two millilitres of the supernatant was transferred into a new 2 ml microcentrifuge tube and the suspended pellet was discarded. One InhibitEX tablet was added to each sample and vortexed immediately and continuously until completely suspended. The suspension was incubated for 1 min at room temperature to allow inhibitors to absorb the InhibitEX matrix. The sample was centrifuged at full speed for 3 min to suspend the inhibitors bound to the InhibitEX matrix and all the supernatant was transferred to a new 1.5 ml microcentrifuge tube while the pellet was discarded. The sample was centrifuged at full speed for 3 min. Fifteen micro-litres of Proteinase K was added into a new 1.5 ml microcentrifuge tube and 200 µl of the supernatant obtained above added to the tube containing the proteinase K. Two hundred micro-litres of buffer AL was added to the mixture and mixed by vortexing for 15 s followed by incubation at 70°C for 10 min. Thereafter, 200 µl of absolute ethanol was added to the lysate and mixed by vortexing. The lid of a new QIAamp spin column was labelled and placed in 2 ml collection tube afterwhich the lysate from above was carefully applied to the QIAamp spin column taking care not to moisten the rim. The transfer was followed by centrifugation at full speed for 1 min. The QIAamp spin column was then placed in a new 2 ml collection tube and the tube containing the filtrate was discarded. A volume of 500 µl of buffer AW1 was added to the QIAamp spin column and centrifuged at full speed for 1 min. The QIAamp spin column was once again placed in a new 2 ml collection tube and the tube containing the filtrate was discarded. Five hundred icro-litres of buffer AW2 was added to the QIAamp spin column and centrifuged at full speed for 3 min. The filtrate containing tube was discarded. The QIAamp spin column was fitted into a new 2 ml collection tube and centrifuged at full speed for 1 min then transferred into a new 1.5 microcentrifuge tube labelled with the final sample code. A volume of 200 µl buffer AE was carefully transferred directly onto the QIAamp membrane and the tube incubated at room temperature for 1 min followed

by 1 min centrifugation at full speed to elute DNA, which was kept at -34°C for future use.

2.2.5 Amplification methods for detection of *H. contortus* DNA

i. Polymerase Chain Reaction (PCR)

Published Real-time PCR primers (Fwd: 5′-catatacatgcaacgtgatgttatgaa-3′ and Rev: 5′-gctcaggttgcattatacaaatgataaa-3′) of Harmon *et al*., (2007) for detection of *H. contortus* were downloaded and used in PCR reactions to confirm the identity of the L_3 that were to be used as positive control. The PCR was performed using 12.5 µl Dream *Taq* Green Mix, 1.0 pmol of each forward and reverse primer, 5.5 µl ddH_2O and 5.0 µl template DNA. The PCR reaction conditions involved an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min.

ii. Loop-mediated isothermal amplification (LAMP)

LAMP was carried out in a thermocycler (Bio-Rad T100^{TM}, Germany). Each 25 µl reaction mixture consisted of 12.5 µl LAMP buffer, 1 µl *Bst* DNA polymerase, 1 µl fluorescent detection reagent (FD), primer mix (0.2 mM of each F3 and B3 primer and 1.6 mM of each FIP and BIP), 6.7 µl ddH_2O and 2.5 µl template DNA.

Table 2: Different PCR reagents used in troubleshooting

Mastermix	Buffer	DNA Polymerase
Taq PCR Mastermix	PCR Buffer (QIAGEN)	HotStar *Taq*
Dream *Taq* Green Mix	Ampdirect Buffer	*Taq* DNA Polymerase
Ampli*Taq* Gold 360		Hot Go *Taq*

2.2.6 Product visualization

Extracted DNA as well as PCR and LAMP products were electrophoresed on a 1% agarose gel (1 g agarose powder and 100 ml TBE buffer) stained with either gel red or ethidium bromide for ~30 min after which visual inspection was done under UV light. A picture of the gel was taken using a gel documentation system (GeneSnap bio imaging system).

2.2.7 Development of LAMP and PCR assay for detection of H. contortus DNA

i. Primer design

Two primer sets targeting the internal transcribed spacer 2 (ITS2) sequence for specific amplification of *H. contortus* gene were designed using the nucleotide sequence with accession number HQ844231.1 downloaded from the NCBI Genbank. The downloaded sequence was uploaded onto LAMP primer design software, PrimerExplorer V4, which generated 5 sets of primers from which, one primer set that annealed closest to the target gene was chosen (**Table 3**). The other set of primers was designed manually to target the same gene. The forward and reverse primers HCF3 and HCB3 respectively and HconF3 and HconB3 respectively were used as PCR primers for specific amplification of *H. contortus* gene.

Table 3: LAMP primer set 1designed manually and primer set 2 designed using primer explorer software

	Primer	Primer Sequence
Primer Set 1	HCF3	5'-ttaatggtggttaagtacgaac-3'
	HCB3	5'-tacgcgaatcaaccgatc-3'
	HCFIP	5'-cgcgtgatcattaaggttcccattacttcttgaagtatgtggtgt-3'
	HCBIP	5'-acagtttgcagaacttagtgttcacatcgctgaagctaatcg a-3'
Primer Set 2	HconF3	5'-gggtaaagtcaagttctt g-3'
	HconB3	5'-cctccgctaaatgatatgc-3'
	HconFIP	5'-gtacgttgcactacaatacgtaaggacttactatacttc-3'
	HconBIP	5'-cagcgggtaatcacgccgctcaggttgcattatac-3'

ii. Optimization of primer annealing temperature

LAMP

To determine a suitable annealing temperature LAMP was carried out in a thermocycler (Bio-Rad T100^{TM}, Germany) wherein both primer sets were tested at the following annealing temperatures 60°C, 63°C and 65°C. The conditions for LAMP were as specified in **Section 2.2.3 ii.** above. From the reactions the optimum annealing temperature of one of the primer sets was determined and used in subsequent reactions.

PCR

PCR was set up to amplify the internal transcribed spacer 2 (ITS2) gene of the *H. contortus* genome in order to determine optimal primer annealing temperature. Several annealing temperatures ranging from 50°C - 60°C were tried and tested together with various incubation periods for each PCR step. Optimization of the newly developed *H. contortus* PCR assay was set up to amplify the internal transcribed spacer 2 (ITS2) gene of the *H. contortus* genome. After optimization, another PCR reaction was set up for screening field samples (few from each sampled province). All PCRs were setup to the volume of 25 µl. Initially several types of PCR reagents and master-mixes (**Table 3**) were used for optimization purposes. Reaction conditions were altered to suite each reaction depending on the denaturation temperature required for activating the enzyme in each mastermix. Several annealing temperatures in the ranges of 50°C, 52°C, 54°C, 56°C 58°C and 60°C were tested for optimal annealing together with various annealing times for each step. The final 25 µl reaction was set up using 12.5 µl *Taq* PCR Mastermix, 6.0 µl of primer mix (20 µl of each forward and reverse primer stock and 60 µl ddH_2O), 1.5 µl ddH_2O and 5.0 µl template DNA. The amplification involved an initial denaturation of 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C for 10 min (using F3 and B3 of primer set 1 **Table 3**).

iii. Optimization of primer specificity

In order to optimise primer sensitivity and specificity DNA was extracted from the adult worms and L_3 by salting-out method as detailed in **Section 2.2.4 i**.

The functional set of LAMP primers and the set derived from the designed LAMP assay for use in PCR were both tested using DNA extracted from closely related species in order to ensure that there was no cross reactivity.

iv. Optimization of primer sensitivity

To determine the detection limit of the assays, *H. contortus* DNA was serially diluted 10 fold. The serial dilutions were then tested by LAMP and PCR at optimum annealing temperatures and reaction times as determined from the above reactions. Of all the samples that were collected in the Free State a few were examined by microscopy and faecal egg counts were conducted. The remaining faecal material

was cultured for cultivation of L_3. The eggs and larvae were collected and placed in separate tubes. DNA was extracted by standard PCI and aliquots of each sample were sent to the NZG for quantification of DNA concentration with a nanodrop spectrophotometer. This would later allow us to at least equate the sensitivity of the developed assays to a certain number of eggs and/or larvae.

2.3 RESULTS

2.3.1 Confirmation of control DNA samples by PCR and LAMP

The DNA extracted from the morphologically identified L_3-Larvae of *H. contortus* were amplified using qPCR primers targeting the ITS2 gene (Harmon *et al*., 2007). The amplification yielded positive results confirming that the larvae were actually of *H. contortus*. Therefore, it was possible to use this DNA as positive control in the subsequent experiments.

2.3.2 Optimisation of LAMP assay

i. Optimisation of primer annealing temperature

Of the two primer sets designed, only primer set 1 gave a positive band when tested at various annealing temperatures. Testing the primer set at various annealing temperatures with optimal operating temperature ranges of LAMP yielded amplification at both 60°C and 63°C. However, optimum primer annealing was achieved at 60°C as can be seen in **Fig 4**.

ii. Primer specificity test

To determine the specificities of the primers, non-target DNA extracted from adult worms of closely related species were used as DNA template. Indeed the amplification did not yield specific bands of the target gene indicating that the primers were not cross-reactive with target DNAs of closely related parasite species (**Fig 5a**).

iii. Primer sensitivity test

When the sensitivity of the primers was determined using seven preparations of ten-fold serial dilutions of *H. contortus* DNA, a detection limit of 0.42 ng was achieved with the target DNA proving that the assay was sensitive (**Fig 6)**. The data that were obtained for the quantified DNA extracted from eggs and larvae (**Fig 3a and b**) was too variable and therefore unreliable and could not be used to determine an equivalent sensitivity for the detection of DNA extracted from the eggs and larvae.

iv. Visualisation of LAMP product

The products of all three LAMP reactions were visualised using three methods, which is an added advantage of LAMP. The visual inspection was based on colour change in reaction tubes containing amplified DNA under normal light (**Fig 5b**) and under fluorescence UV light (**Fig 5c**), as well as on 1% agarose gel stained with gel red.

2.3.3 Optimisation of PCR assay

i. Optimization of PCR conditions

Several attempts to optimise the primer set for annealing temperature resulted in amplification of *H. contortus* DNA. Amplification were observed at an annealing temperature of 55°C at the following PCR conditions: 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C for 10 min.

ii. Primer specificity test

After establishing the optimum annealing temperature, the specificity of the assay was determined by testing the primer set using DNA extracted from closely related parasite species. The test yielded a specific band of target DNA without any cross reaction with any of the DNA samples extracted from closely related species. The data is represented in **Fig 7** below.

iii. Primer sensitivity test

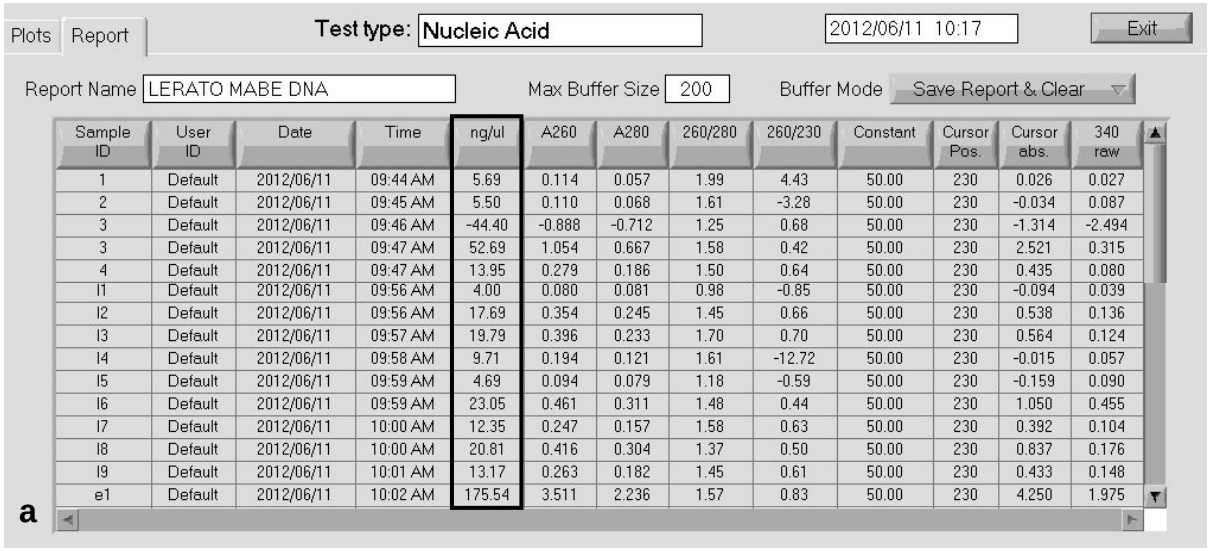
The sensitivity of the primer set was evaluated using eight preparations of ten-fold serial dilutions of *H. contortus* DNA. The sensitivity test yielded a detection limit of 10^{-3} ng as shown in **Fig 6** below.

iv. Product visualization

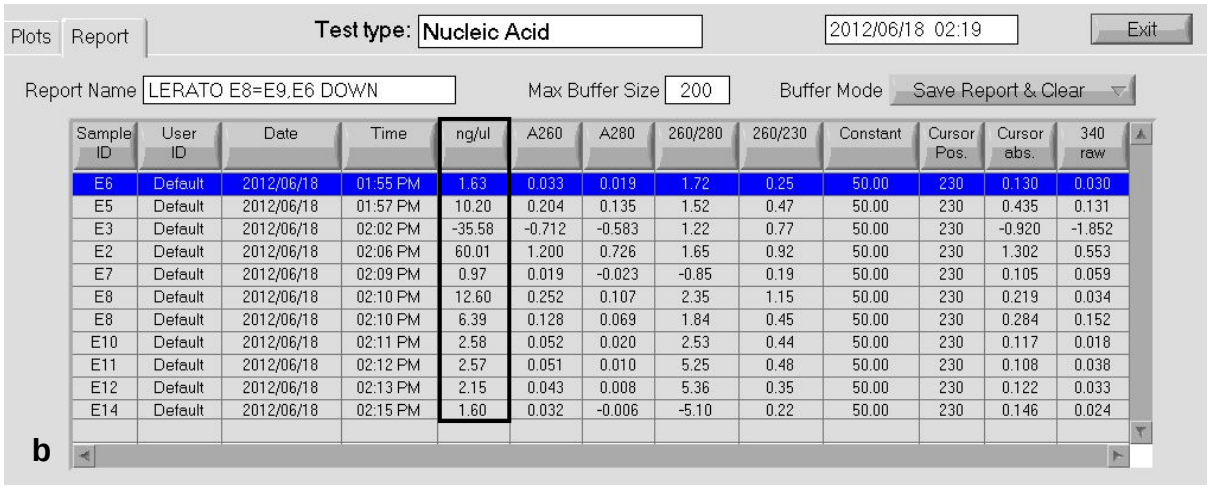
The PCR products of the both the optimization of primer specificity and sensitivity were analysed on 1% agarose gel electrophoresis stained with gel red. The results were captured using a gel documentation system and are shown in the **Figures 4, 5a, 5b, 5c, 6** and **7** as stated above.

2.3.4 Screening field samples by PCR

In order to validate the assay, PCR was setup at optimised conditions. DNA extracted from faecal samples collected in various provinces of South Africa was screened for the presence of *H. contortus* DNA. Three reactions were setup in which a total of 22 randomly selected DNA samples extracted from faecal samples obtained from 22 individuals were screened. The first PCR reaction was setup with DNA extracted using the PCI method, the second reaction was setup with DNA extracted using the salting out method while the final reaction was setup with DNA samples extracted using the QIAamp Mini Stool kit. The PCR assay yielded negative results in all the screened samples for all of the reactions. However, the positive control sample was amplified and yielded positive results as expected in all the reactions. The results of the latter reactions led to the setup of a second round of reactions in which the DNA extracted from each faecal sample was spiked with a 1µl aliquot of the target DNA extracted from laboratory identified *H. contortus* larvae which were later verified as suitable for use as positive control. The DNA was spiked to determine if there were any faecal inhibitors in the faecal extracts that were hindering the amplification of DNA or if the lack of amplification was due to the poor quality or absence of parasite DNA in the extracts. The spiked DNA extracts were then re-screened by PCR and the products separated on 1% agarose gel stained with ethidium bromide. Only a single band of amplified DNA belonging to the positive control was observed as before.



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	2012/06/11	09:44 AM	5.69	0.114	0.057	1.99	4.43	50.00	230	0.026	0.027
2	Default	2012/06/11	09:45 AM	5.50	0.110	0.068	1.61	-3.28	50.00	230	-0.034	0.087
3	Default	2012/06/11	09:46 AM	-44.40	-0.888	-0.712	1.25	0.68	50.00	230	-1.314	-2.494
3	Default	2012/06/11	09:47 AM	52.69	1.054	0.667	1.58	0.42	50.00	230	2.521	0.315
4	Default	2012/06/11	09:47 AM	13.95	0.279	0.186	1.50	0.64	50.00	230	0.435	0.080
l1	Default	2012/06/11	09:56 AM	4.00	0.080	0.081	0.98	-0.85	50.00	230	-0.094	0.039
l2	Default	2012/06/11	09:56 AM	17.69	0.354	0.245	1.45	0.66	50.00	230	0.538	0.136
l3	Default	2012/06/11	09:57 AM	19.79	0.396	0.233	1.70	0.70	50.00	230	0.564	0.124
l4	Default	2012/06/11	09:58 AM	9.71	0.194	0.121	1.61	-12.72	50.00	230	-0.015	0.057
l5	Default	2012/06/11	09:59 AM	4.69	0.094	0.079	1.18	-0.59	50.00	230	-0.159	0.090
l6	Default	2012/06/11	09:59 AM	23.05	0.461	0.311	1.48	0.44	50.00	230	1.050	0.455
l7	Default	2012/06/11	10:00 AM	12.35	0.247	0.157	1.58	0.63	50.00	230	0.392	0.104
l8	Default	2012/06/11	10:00 AM	20.81	0.416	0.304	1.37	0.50	50.00	230	0.837	0.176
l9	Default	2012/06/11	10:01 AM	13.17	0.263	0.182	1.45	0.61	50.00	230	0.433	0.148
e1	Default	2012/06/11	10:02 AM	175.54	3.511	2.236	1.57	0.83	50.00	230	4.250	1.975



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
E6	Default	2012/06/18	01:55 PM	1.63	0.033	0.019	1.72	0.25	50.00	230	0.130	0.030
E5	Default	2012/06/18	01:57 PM	10.20	0.204	0.135	1.52	0.47	50.00	230	0.435	0.131
E3	Default	2012/06/18	02:02 PM	-35.58	-0.712	-0.583	1.22	0.77	50.00	230	-0.920	-1.852
E2	Default	2012/06/18	02:06 PM	60.01	1.200	0.726	1.65	0.92	50.00	230	1.302	0.553
E7	Default	2012/06/18	02:09 PM	0.97	0.019	-0.023	-0.85	0.19	50.00	230	0.105	0.059
E8	Default	2012/06/18	02:10 PM	12.60	0.252	0.107	2.35	1.15	50.00	230	0.219	0.034
E8	Default	2012/06/18	02:10 PM	6.39	0.128	0.069	1.84	0.45	50.00	230	0.284	0.152
E10	Default	2012/06/18	02:11 PM	2.58	0.052	0.020	2.53	0.44	50.00	230	0.117	0.018
E11	Default	2012/06/18	02:12 PM	2.57	0.051	0.010	5.25	0.48	50.00	230	0.108	0.038
E12	Default	2012/06/18	02:13 PM	2.15	0.043	0.008	5.36	0.35	50.00	230	0.122	0.033
E14	Default	2012/06/18	02:15 PM	1.60	0.032	-0.006	-5.10	0.22	50.00	230	0.146	0.024

Fig 3: DNA concentrations measured using a nanodrop spectrophotometer. **a** (1) 6 Eggs, (2) 11 Eggs, (3) 1 Egg, (4) 1 Egg, (l1) 10 L3, (l2) 30 L3, (l3) +50 L3, (l4) +100 L3, (l5) 7 L3, (l6) 15 L3, (l7) 20 L3, (l8) 35 L3, (l9) +100 L3, (e1) 6 Eggs. **b** (E6) 87 Eggs, (E7) 1 Egg, (E8) 9 Eggs, (E9) 18 Eggs, (E10) 3 Eggs, (E11) 2 Eggs, (E12) 26 Eggs, (E14) 6 Eggs

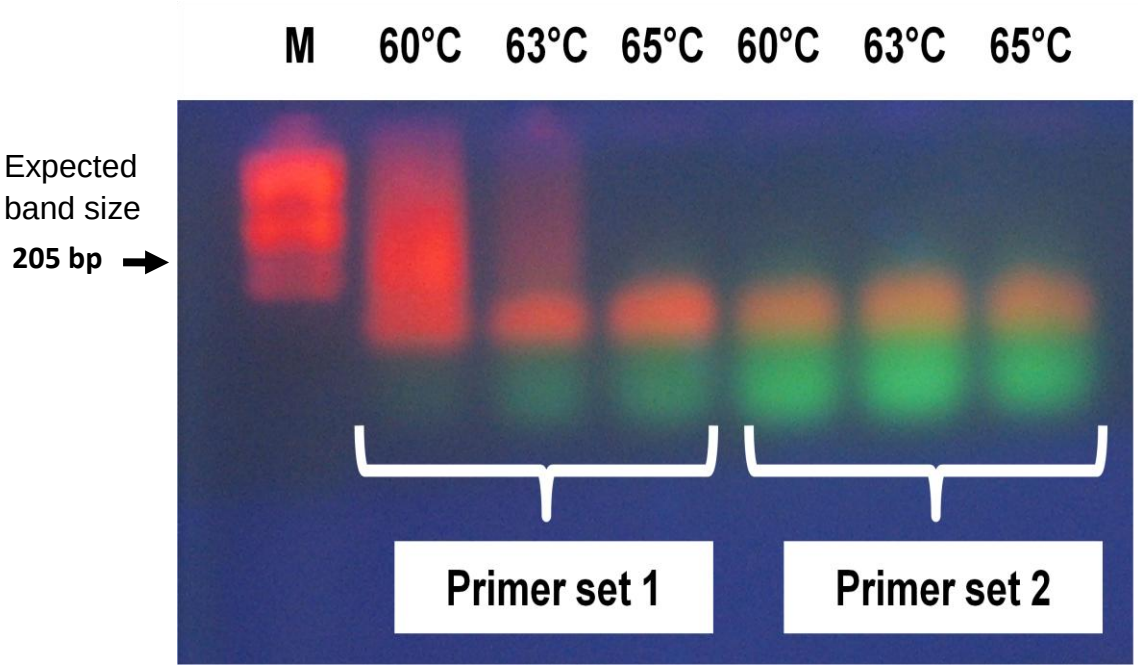


Fig 4: Agarose gel electrophoresis (1%) of *H. contortus* DNA amplified at different primer annealing temperatures. The results show the efficacy of each primer set. (M) 1kbp Molecular marker.

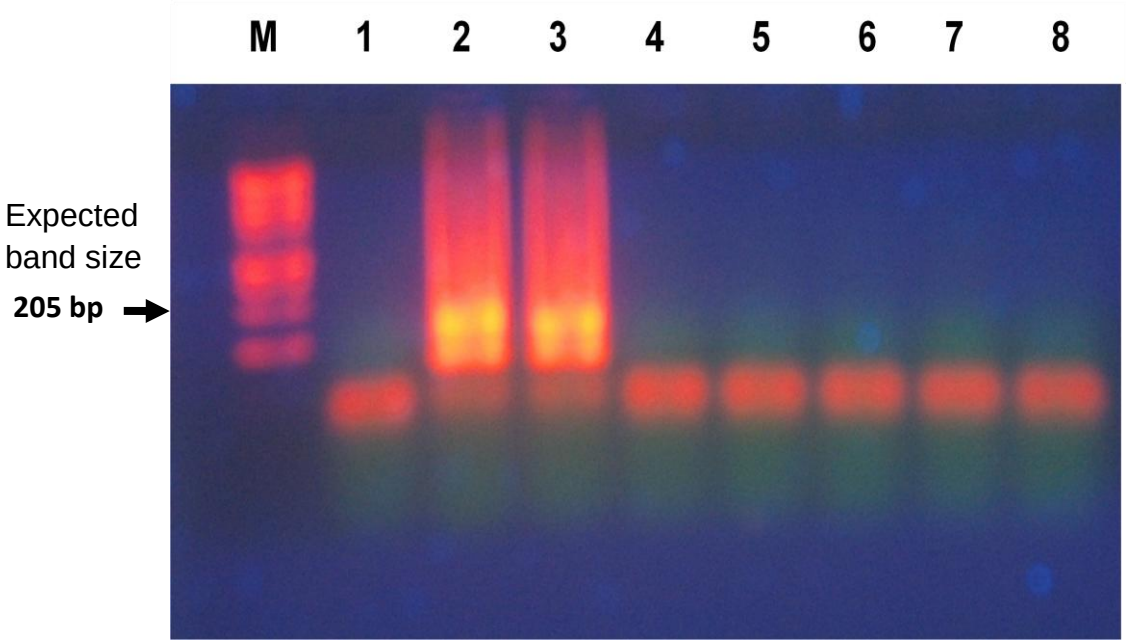


Figure 5a: Agarose gel electrophoresis (1%) showing specific amplification of *H. contortus* DNA by LAMP, amongst closely related species. (M) 100 bp Molecular marker, (1) ddH_2O, (2) *H. contortus*, (3) *H. contortus*, (4) *H. placei*, (5) *F. hepatica*, (6) *Paramphistomum* sp., (7) *Taenia saginata*, (8) Bovine faecal sample.

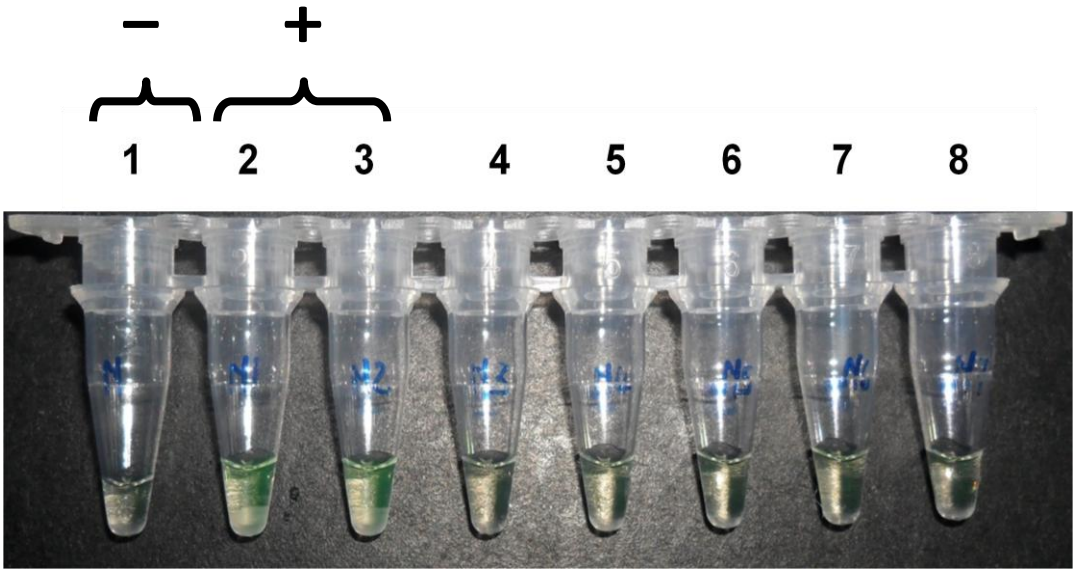


Figure 5b: Visual inspection of LAMP amplification products under normal light. The specific amplification of target DNA of *H. contortus* and lack of amplification for closely related species are shown. (1) ddH_2O, (2) *H. contortus*, (3) *H. contortus*, (4) *H. placei*, (5) *F. hepatica*, (6) *Paramphistomum* sp., (7) *Taenia saginata*, (8) Bovine faecal sample.

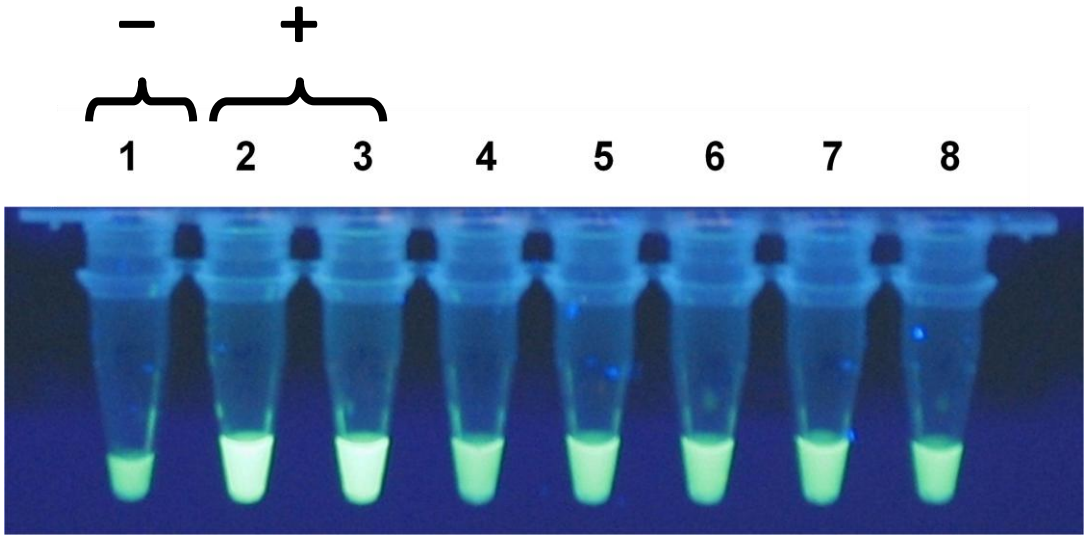


Figure 5c: Visual inspection of LAMP amplification products under UV light. The specific amplification of target DNA of *H. contortus* and lack of amplification for closely related species are shown. (1) ddH_2O, (2) *H. contortus*, (3) *H. contortus*, (4) *H. placei*, (5) *F. hepatica*, (6) *Paramphistomum* sp., (7) *Taenia saginata*, (8) Bovine faecal sample.

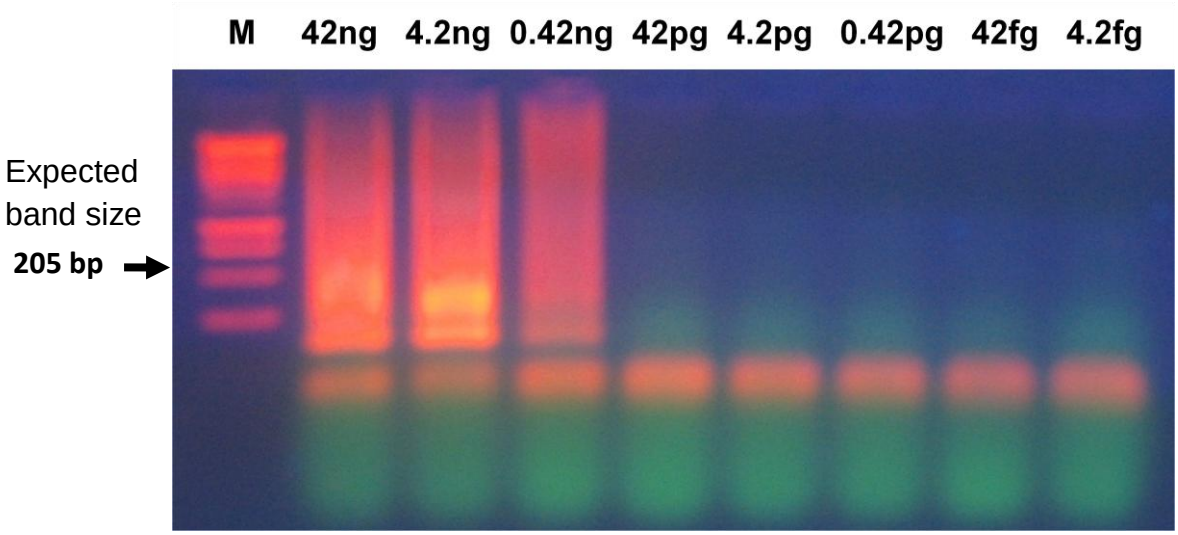


Fig 6: Agarose gel electrophoresis (1%) of LAMP assay for detection of *H. contortus* DNA showing the detection limit of the assay. (M) 100 bp Molecular marker.

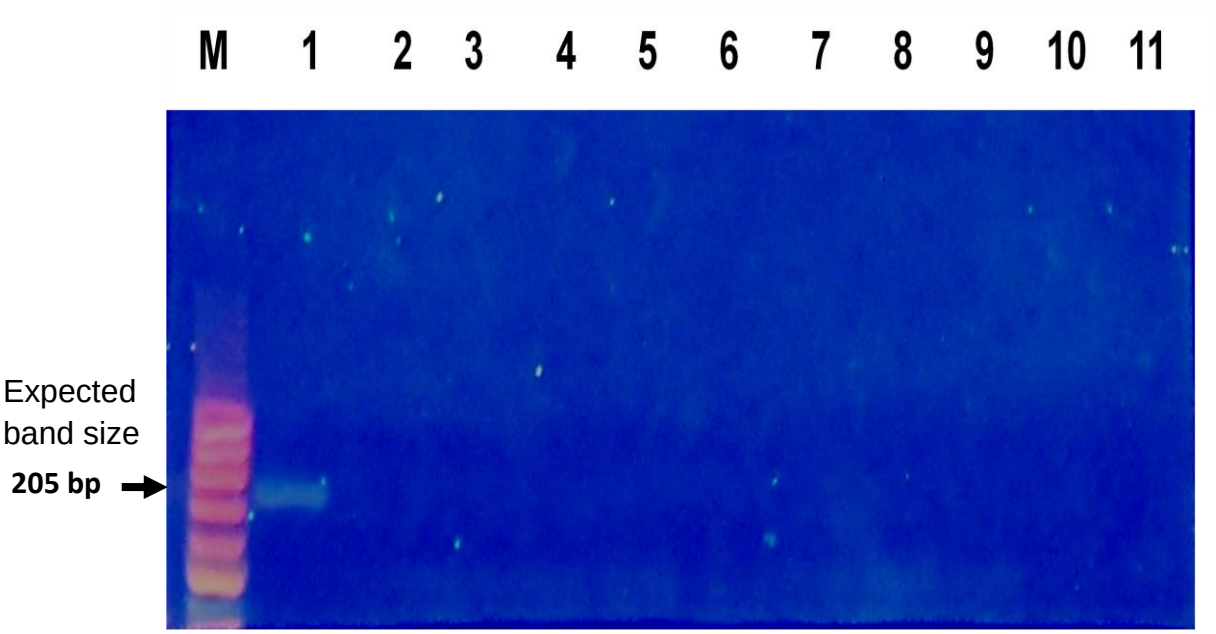


Fig 7: Agarose gel (1%) stained with gel red after amplification by PCR assay and showing specific amplification of *H. contortus* DNA and lack of amplification for target genes of closely related parasite species. (M) 100 bp Molecular marker, (1) *H. contortus*, (2) *H. placei*, (3) *F. hepatica*, (4) *Trichostrongylus* spp., (5) *Paramphistomum* spp., (6) *Cooperia* spp., (7) *Schistosoma mattheei*, (8) *Oesophagostomum radiatum*, (9) *Avitellina* spp., (10) *Trichuris* spp., (11) ddH_2O.

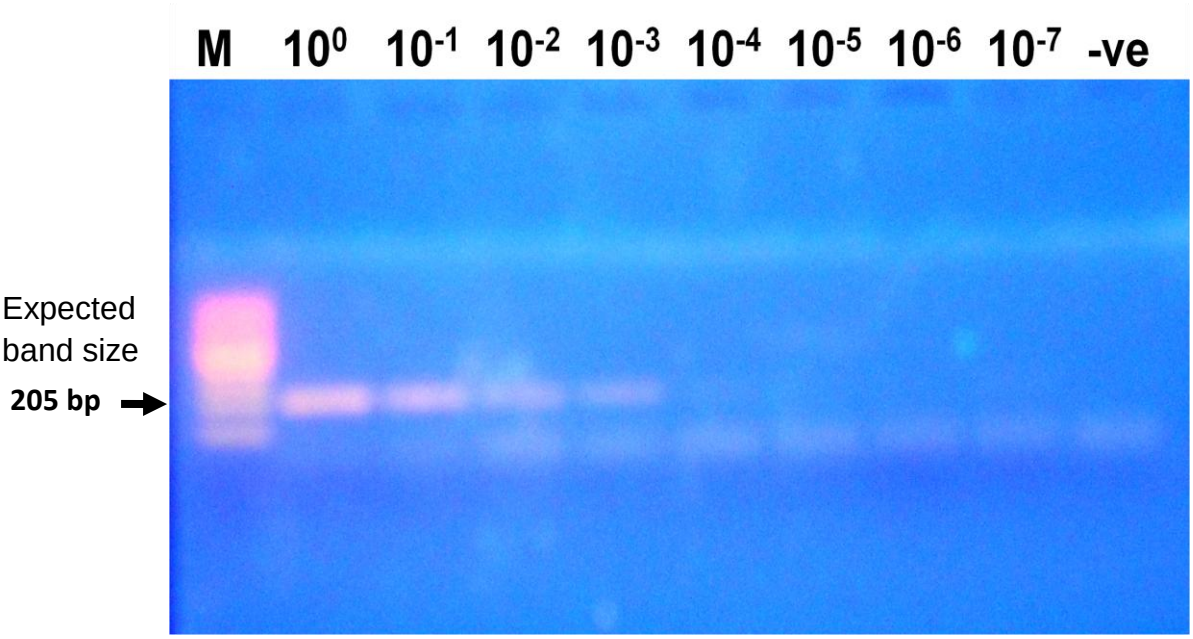


Figure 8: Agarose gel (1%) stained with gel red after amplification by PCR assay. The specific bands of *H. contortus* target DNA show the detection limit of the assay. (M) 100 bp Molecular marker, (-ve) ddH_2O.

2.4 DISCUSSION AND CONCLUSIONS

Haemonchus contortus is reported as one of the most prevalent species of nematodes in livestock (von Samson-Himmelstjerna *et al*., 2002). Adults inhabit the abomasum of ruminants where they feed on blood and cause disease. Infections with this nematode species result in reduced production of meat, milk and wool resulting in lower profit margins in the livestock industry (von Samson-Himmelstjerna *et al*., 2002). Traditionally diagnosis is based on examination of morphological character of adult or juvenile stages as well eggs. This method of diagnosis is lacking in sensitivity and specificity due to subtle morphological differences that exist amongst closely related species (von Samson-Himmelstjerna *et al*., 2002; Schnieder *et al*., 1999; Harmon *et al*., 2007). Recent advances in molecular diagnostics have lead to development of reliable diagnostic tools for detection of *H. contortus* (Schnieder *et al*., 1999; von Samson-Himmelstjerna *et al*., 2002; Learmount *et al*., 2009).

The present study resulted in successful development of LAMP and PCR assays for detection of *H. contortus* infections. The assay was validated using the L_3 stage of *H. contortus*, these were cultivated under laboratory conditions and microscopically identified. When subjected to a series of tests both assays were able to discriminate target from non-target DNA. The expected LAMP and PCR products were amplified and there was no cross reaction between the reagents and non-target DNA. This means that the assays can be used reliably in mixed infections. The assays also detected target DNA at concentration as low as 0.42 ng and 10^{-3} ng for LAMP and PCR respectively. Further quantification of the sensitivity of each assay could not be achieved because of the variable and unreliable concentrations of the parasite DNA. This variation could be as a result of the presence of non-target DNA derived from faecal material. Despite this problem it is possible that the sensitivity of the assays could be improved by the addition of detergent to PCR reagents during the setup process and this is recommended for future studies. The development of molecular diagnostic tools such as the PCR and LAMP is vital for rapid, sensitive and specific detection of infections as well as for evaluating drug efficacy (Schnieder *et al*., 1999; Harmon *et al*., 2007).

Attempts to validate the PCR assay using samples collected from the field were unsuccessful on several occasions. Nevertheless, the same primers that gave negative results with the field samples successfully amplified the target DNA in positive control samples. Perhaps poor quality or even the lack of target DNA could have contributed to the negative results that were observed with the faecal samples obtained from the field. Further attempts to improve on the DNA extraction of all the field samples by using PCI, Salting-out and QIAamp DNA stool kit yielded negative results on PCR. The reason for no amplification is believed to be the presence of faecal inhibitors in the PCR reaction mixture as spiking DNA samples with control DNA also yielded negative results. Due to time constraints we did not get the chance to address these. In another study, Harmon *et al*., (2007) treated the eggs extracted from faecal material with phytase before DNA extraction. They showed that faecal inhibitors may be present but only in amounts sufficient enough to affect amplification if small amounts of DNA are present to begin with. Therefore it seems that methods of DNA extraction used in this study may not have provided sufficient disruption of the nematode eggs to allow sufficient amounts of DNA to be released. Karajeh *et al*., (2010) pointed out the importance of the duration of initial incubation for Proteinaise K digestion as this will determine the amount of Proteinase K that gets into the eggs and thus disrupting them in order to release DNA molecules. In light of this information, it is possible that the incubation of faecal samples for Proteinase K digestion of the faecal eggs was not long enough to allow proper disruption of the eggs.

Validation of the assay by LAMP using the same field samples was not attempted as possible faecal inhibitors were established in the DNA. Subsequent reactions that were setup as a form of positive control yielded negative results. One of the suspected reasons for the abrupt failure of the PCR assay was a problem with primer synthesis by the supplying company since a new batch of primers that had just been ordered failed to work. In future, it may be important to validate sensitive and rapid PCR and LAMP assays for their possible use in diagnosing natural infections with this important parasitic nematode.

CHAPTER 3

DEVELOPMENT OF LAMP AND PCR ASSAYS FOR DETECTION OF *TRICHOSTRONGYLUS* SPECIES

3.1 INTRODUCTION

3.1.1 Introduction and classification of *Trichostrongylus* spp.

Kingdom: Animalia
Phylum: Nematoda
Class: Chromadorea
Order: Rhabditida
Family: Trichostrongylidae
Genus: *Trichostrongylus*

Trichostrongylosis also known as trichostrongyliasis is a disease of herbivorous animals (Yong *et al*., 2007) such as cattle, sheep and goats. This disease is caused by the nematodes of the genus *Trichostrongylus* commonly known as the small intestinal hair worm, bankrupt worm and the black scours worm (Kaufmann, 1996; Wall *et al*., 2011). These causative agents are often found in mixed infections with other gastrointestinal nematodes, including *Haemonchus contortus* and *Ostertagia* species (Kaufmann, 1996; Sato, 2011). This makes it difficult to accurately determine the damage caused by infections except that they add to primary infections (Kaufmann, 1996).

Trichostrongylus species are distributed worldwide; however, they are more densely distributed in warmer parts of the world (Kaufmann, 1996; Yong *et al*., 2007; Lattès *et al*., 2011; Sato *et al*., 2011). Described species include *T. orientalis*, *T. colubriformis*, *T. probolurus*, *T. vitrinus*, *T. capricola*, *T. axei* and *T. skrjabini* (Kaufmann, 1996 and Yong *et al*., 2007). They are identifiable as “small reddish-brown worms without a specially developed head end” (Kaufmann, 1996). In the host the parasites occupy the small intestines and feed on host blood (Lattès *et al*., 2011).

Cases of human trichostrongylosis have been reported in several countries despite the difficulty in identifiying them due to lack of symptoms and accurate methods of detection (Yong *et al*., 2007; Lattès *et al*., 2011; Sato *et al*., 2011). The prevelance of

infection is particularly high in developing countries where there is no proper sanitation with increased contact between man and livestock (Wall *et al*., 2011). In China, Korea, Japan and Armenia *T. orientalis* is the most frequently encountered species of *Trichostrongylus* in humans. On the other hand, all known species of *Trichostrongylus* have been reported in Iran with *T. orientalis* and *T. colubriformis* having been reported as the most common parasites (Yong *et al*., 2007; Sato *et al*., 2011). Often *Trichostrongylus* spp. are mistaken for hookworms because of similarities that exist in their morphology, modes of transmission and pathophysiology (Yong *et al*., 2007). To illustrate this point, in a study conducted by Sato *et al*., (2011), faecal samples were collected from 253 individuals in a rural village in Laos and examined microscopically for the presence of nematode eggs (more specifically *Trichostrongylus* eggs). Of the 253 samples that were examined, 125 tested positive for hookworm eggs and out of these 125 individuals, 46 volunteered to be used as guinea pigs in the study for further recovery of adult worm. Out of these 46 volunteers, 43 actually tested positive for *Trichostrongylus* infection whilst only three were actually positive for hookworm infection. But even in these three cases the recovery of adult worms showed mixed infections with *Trichostrongylus* spp. and hookworms.

According to Yong *et al*., (2007), there is a scarcity of information regarding *Trichostrongylus* spp. and this scarcity is a direct result of "the difficulty inherent to the differentiation of *Trichostrongylus* from the 2 most important human hookworm species, *Ancylostoma duodenale* and *Necator americanus*," based on their morphology at different stages of their life cycles.

3.1.2 Life cycle of *Trichostrongylus* spp.

Adult females in the small intestines of the definitive host (ruminants) produce eggs that are passed out in the faeces. When environmental conditions such as moisture, warmth and shade are favourable the eggs hatch into first stage larvae within several days. The larvae mature into second stage larvae in the soil or on vegetation, and after 5 to 10 days (and two molts) they mature into the infective third-stage larvae known as the filariform larvae. Human hosts become infected by ingestion of vegetables contaminated with the infective stage of the parasite (Sato *et al*., 2011) as well as water contaminated by animal faeces (Wall *et al*., 2011). After ingestion

the larvae make their way into the small intestines where they mature into adults. Adult worms inhabit the digestive tract of their definitive hosts and may occur as incidental infections in humans. Life cycle (**Fig 9**) extracted from Lattès *et al*., (2011) and http://www.dpd.cdc.gov/dpdx.

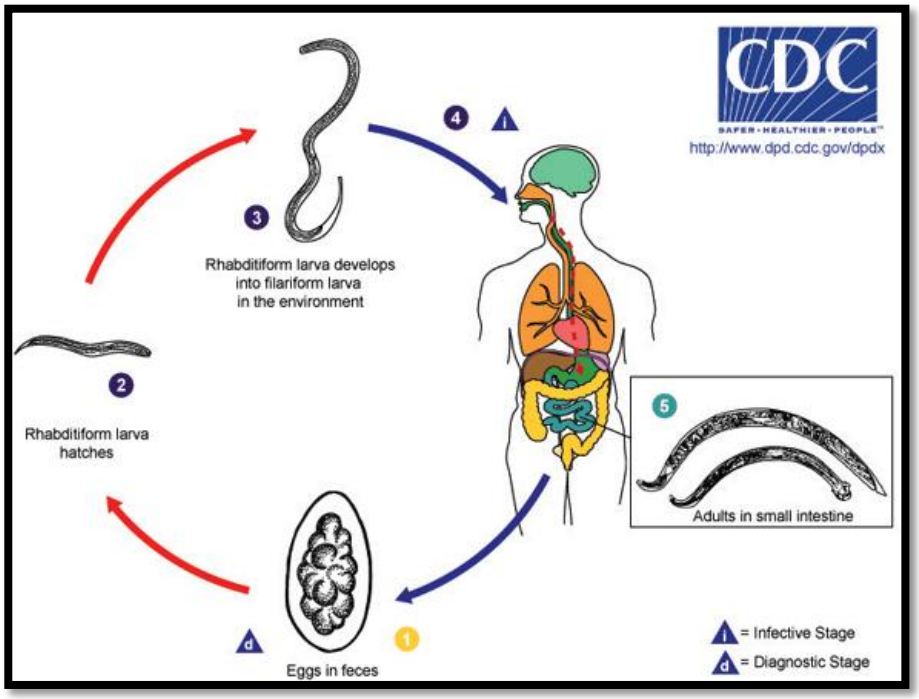


Fig 9: Life cycle of *Trichostrongylus* spp. Figure extracted from http://www.dpd.cdc.gov/dpdx

3.1.3 Diagnosis of *Trichostrongylus* spp. infections

Mild infections are often asymptomatic in both human hosts and ruminants. However, in heavy infections clinical symptoms such as stomach aches, abdominal bloating, weakness, weight loss, reduced appetite, diarrhoea or constipation may be observed (Andrews, 1939; Kaufmann, 1996; Lattès *et al*., 2011; Wall *et al*., 2011). Diagnosis is mainly based on microscopic examination of faecal samples for eggs (Kaufmann, 1996; Lattès *et al*., 2011; Sato, 2011). *Trichostrongylus* eggs can be differentiated from those of closely related nematodes by their size and shape (Wall *et al*., 2011). This can be done by direct examination using the formalin-ether concentration technique (Yong *et al*., 2007) and the Merthiolate–iodine–formaldehyde concentration technique. However, identification and differentiation of various *Trichostrongylus* spp. eggs from those of hookworms in the faeces has proved quiet difficult due to morphological similarities that exist between the eggs of these helminths (Yong *et al*., 2007; Lattès *et al*., 2011). As a result egg recovery and identification is complemented with larval culture for identification of L_3 larvae (Hoste *et al*., 1995). However, even with culture of larval stages the identification of larvae to species level is still unreliable and laborious (Hoste *et al*., 1995).

Infections with *Trichostrongylus* spp. can also be diagnosed by immunological method. This method of diagnosis is based on the detection of the presence or absence of antibodies against certain parasites in the host. However, these immunological assays are considered only a tiny fragment more reliable than microscopic tests (Hoste *et al*., 1995).

Breakthroughs in molecular technology have resulted in the development of PCR assays for specific amplification of *Trichostrongylus* spp. DNA. Hoste *et al*., (1995) reported on PCR amplification of *Trichostronylus* spp. using primers NC1: 5′-acgtctggttcagggttgtt-3′ (forward) and NC2: 5′-ttagtttcttttcctccgct-3′ (reverse). Due to difficulties inherent to the differentiation of *Trichostrongylus* and hookworms infections Yong *et al*., (2007) reported on a PCR assay that differentiates *Ancylostoma duodenale, Necator americanus and Trichostrongylus colubriformis* with reliability. The latter discussed detection and differentiation of the three species is validated on column-purified eggs (Bott *et al*., 2009), using third stage larvae that have been cultivated from faecal samples or using adult worms recovered from the

host (Hoste *et al*., 1995) and not directly on the faeces. However, the possibility of such advances for future research was acknowledged although there are concerns about the accuracy of the diagnosis due to uneven distribution of the eggs in the faeces and the small quantity of faeces that are used during DNA extraction (Sweeny *et al*., 2012). Sweeny *et al*., (2011) demonstrated that molecular assays can potentially give accurate diagnostic results using DNA extracted directly from faeces. They found the difference in accuracy to detect pathogens between microscopic faecal egg counts and molecular techniques to be a mere 7%. The latter study used previously described primers TRI - NC2 (Bott *et al*., 2009) for specific amplification of *Trichostrongylus* spp. from DNA extracted directly from faeces.

3.1.4 Objectives

- To develop a LAMP assay for detection of *Trichostrongylus* species infections in cattle, sheep and goats.
- To develop a PCR assay for detection of *Trichostrongylus* species infections in cattle, sheep and goats.
- To apply the newly developed LAMP and PCR assays in determining prevalences of infections with *Trichostrongylus* species.

3.2 MATERIALS AND METHODS

3.2.1 Helminth samples used for positive and negative control purposes

Adult worms used in this study (*Trichostrongylus* spp., *Haemonchus contortus*, *H. placei*, *Fasciola hepatica*, *F. gigantica*, *Trichuris spp.*, *Oesophagostomum radiatum*, *Schistosoma mattheei*, *Cooperia* spp., *Avitellina* spp., and *Paramphystomum* spp.) were collected, preserved and their DNA extracted as detailed in **Chapter 2 Section 2.2.1**.

3.2.2 Faecal sample collection and storage

Fresh faecal samples were collected from cattle, sheep and goats in four South African Provinces. The samples were processed and preserved as stated in **Chapter 2 Section 2.2.2**.

3.2.3 Methods used for DNA extraction and troubleshooting

DNA was extracted from the eggs, the larvae, adult worms and from faecal samples by the salting out method. In order to troubleshoot, two other methods of DNA extraction were tested for extraction of DNA from the adult worms and faecal samples respectively. The PCI method was further modified by addition of either 1%, 1M, 0.5M and 0.05M of 2-mecarptoethanol to the extraction buffer containing worm tissue samples to assist with disruption of the adult worms. The different methods of extraction are detailed in **Chapter 2 Section 2.2.4**.

3.2.4 Amplification methods for detection of *Trichostrongylus* spp. DNA

i. PCR

All PCRs were setup to the volume of 25 µl. The PCR mixture used for amplification of DNA consisted of 2.5 µl PCR buffer (QIAGEN), 0.5 µl (5mM) dNTPs, 10 pmol of each forward and reverse primer, 0.1 µl HotStar *Taq* DNA polymerase, 1.0 µl BSA, 15.9 µl ddH_2O and 3.0 µl template DNA. Reactions were initially incubated in a MultiGene OptiMax Thermal Cycler (Life Technologies, USA) at the following PCR conditions: 95°C for 15 min followed by 35 cycles of amplification at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 10 min. Several types of PCR master-mixes, buffers and DNA polymerases were used for troubleshooting (**Table 3, Chapter 2**) while reaction condition were altered to suite each reaction depending on the denaturation temperature required for activating the

enzyme in each mastermix. Reagent concentrations and volumes were also altered a number of times and several annealing temperatures ranging from 50°C - 60°C were tried and tested together with different incubation periods for each step.

ii. Troubleshooting of PCR assay

PCR primers that are already publishes, jhTsp (5'- ttatgtgccacaaatgaaga-3') and NC2 (5′-ttagtttcttttcctccgct-3′) (Yong *et al*., 2007) were retrieved and used for troubleshooting the PCR using DNA samples extracted by employing the different methods. A 25 µl reaction was set up using 12.5 µl Dream *Taq* Green Mix, 10 pmol of each forward and reverse primer, 5.5 µl ddH_2O and 5.0 µl template DNA. The amplification conditions for the PCR were as follows: 95°C for 15 min followed by 30 cycles of 94°C for 1 min, 52°C for 40 s and 72°C for 90 s and a final extension step of 72°C for 15 min.

iii. LAMP

All reactions were done using 25 µl PCR mixtures consisting of 12.5 µl LAMP buffer, 1 µl *Bst* DNA polymerase, 1 µl fluorescent detection reagent (FD), primer mix (0.2 mM of each F3 and B3 primer and 1.6 mM of each FIP and BIP), 2.5 µl template DNA. The final volume was adjusted to 25 µl by adding ddH_2O.

3.2.5 Product visualization

All DNA extractions, PCR and LAMP products were visualized on 1% agarose gel electrophoresis stained with ethidium bromide or gel red, under UV light and gel documentation was done using GeneSnap bio imaging system.

3.2.6 Development of LAMP and PCR assay for detection of *Trichostrongylus* spp.

i. Primer design

Two primer sets targeting the Internal transcribed spacer 2 (ITS2) gene for genus-specific amplification of *Trichostrongylus* spp. were designed using the nucleotide sequence with accession number HQ844229. The sequence was downloaded from the NCBI DNA gene database and then uploaded onto LAMP primer design software, PrimerExplorer V4. This software generated five sets of primers from which

two sets that annealed closest to the target gene were chosen (**Table 4**). From each primer set, F3 and B3 primers were used as PCR primers for genus-specific amplification.

Table 4: Designed LAMP assays for genus-specific detection of *Trichostrongylus* spp.

Primer	***Trichostrongylus* spp.(ITS)**	***Trichostrongylus* spp.(Tsp)**
F3	5′-aaagtttgcagaaacgtgt-3′	5′-tgagcttgagacttaatgagt-3′
B3	5′-agagttagccacactgtag-3′	5′-gaagctagtcttcatttgtgg-3′
FIP	5′-tcgatacgcgaatcgaccgctattt atgtgccacaaatgaaga-3′	5′-acatacaacaaggaaatgttgcgta attgcaataataccgcctca-3′
BIP	5′-gcagacgcttagagtggtgaaac aaccctgaaccagac-3′	5′-ggaaaccttaatgatctcgcctagc acacgtttctgcaaact-3′

ii. Optimization of the assay

Primer annealing temperature

A number of LAMP reactions were setup and ran in a Loopamp Turbidimeter (Teramecs, Japan) at various conditions in order to find suitable conditions for amplification. Temperatures ranging from 60°C - 65°C as well as incubation time of 60 min - 120 min were explored.

A PCR method targeting the internal transcribed spacer 2 (ITS2) gene of *Trichostrongylus* spp. was used to determine optimal conditions for primer annealing temperature.

3.3 RESULTS

3.3.1 Optimisation of LAMP assay

To evaluate the efficacy of the designed assay for testing genus-specific detection of *Trichostrongylus* spp., LAMP was conducted using a range of annealing temperatures. The primer set, however, did not yield any positive amplification at any of the tested annealing temperatures or at extended incubation periods. The figure below (**Fig 10**) is a typical representation of the results of each reaction.

3.3.2 Optimisation of PCR assay

Efforts to amplify the internal transcribed spacer 2 (ITS2) gene of the *Trichostrongylus* spp. genome by PCR were unsuccessful. A series of reactions setup to determine optimal conditions for primer annealing gave negative results. Various PCR conditions were tested as well as different reagents, reagent volumes and concentrations and DNA samples extracted by PCI, salting out and by a DNA extraction kit. However, the primers failed to amplify target DNA after all efforts had been exhausted. The figure below (**Fig 11**) shows typical results after each reaction was conducted.

3.3.3 Troubleshooting of PCR assay

A PCR that targets the ITS1 gene was setup, for genus-specific amplification of *Trichostrongylus* spp., using primers previously published by Yong *et al*., (2007). Several amplification attempts with samples extracted using various extraction methods were unsuccessful yielding negative results in all instances.

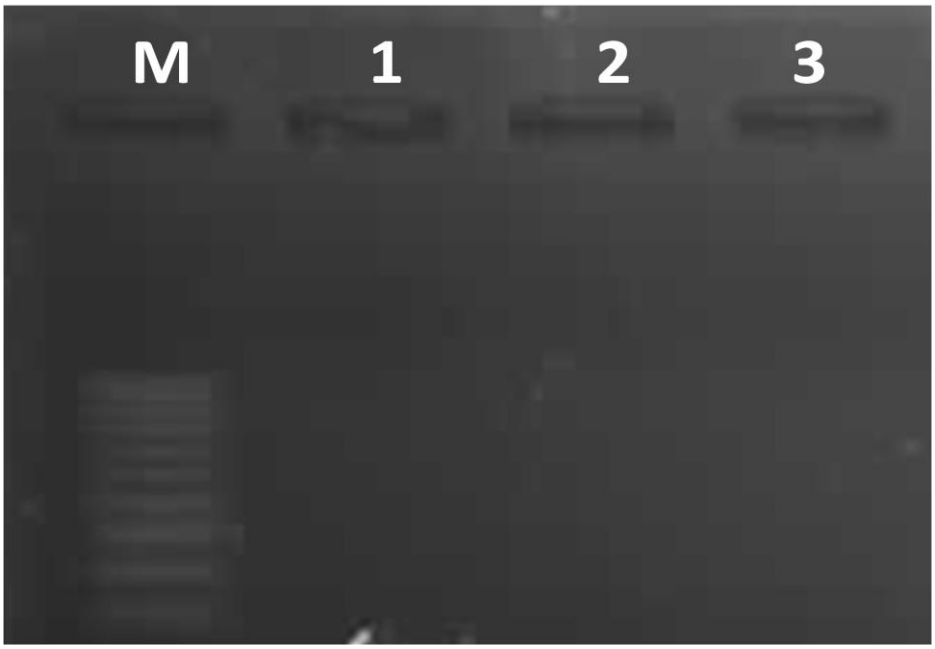


Fig 10: Agarose gel electrophoresis (1%) of LAMP assay for genus-specific detection of *Trichostrongylus* spp. (M) 100 bp Molecular marker, (1) ddH_2O, (2) *Trichostrongylus* spp., (3) *Trichostrongylus* spp.

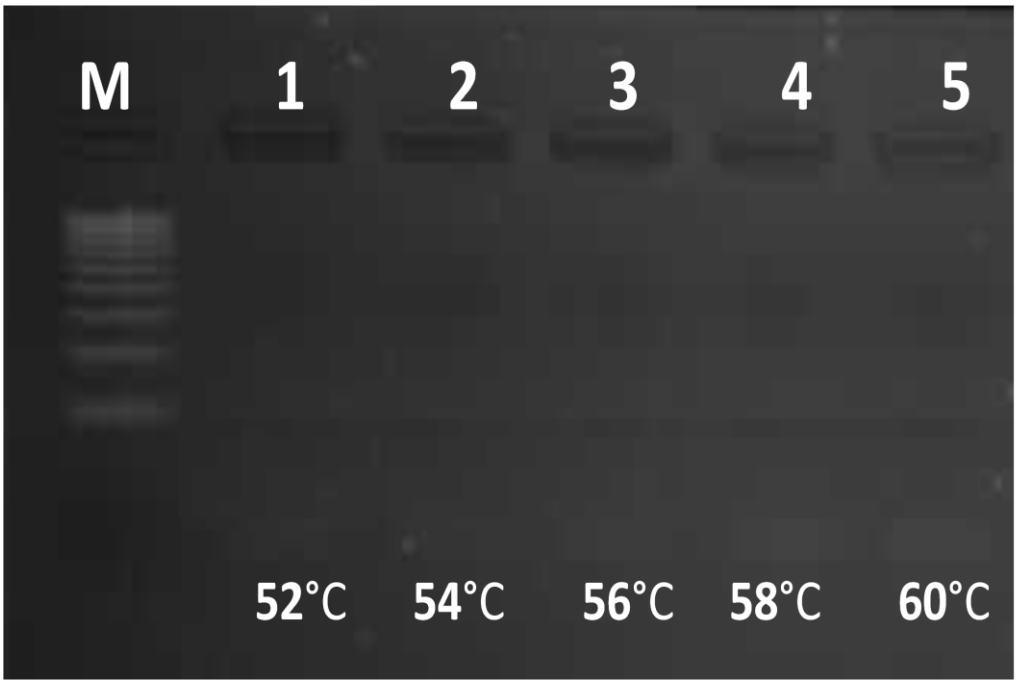


Fig 11: Agarose gel electrophoresis (1%) of PCR assay for genus-specific detection of *Trichostrongylus* spp. at different annealing temperatures. (M) 100 bp Molecular marker.

3.4 DISCUSSION AND CONCLUSIONS

Trichostrongylus spp. are often found in mixed infections with other gastrointestinal nematodes and therefore contribute to the deteriorating health conditions of livestock (Kaufmann, 1996; Sato *et al*., 2011). Infections are often asymptomatic and difficult to diagnose as the worms are often mistaken for hookworms due to morphological similarities, modes of transmission and pathophysiology (Yong *et al*., 2007; Lattès *et al*., 2011). Despites these challenges human infections have been reported in several countries around the globe (Yong *et al*., 2007; Lattès *et al*., 2011; Sato *et al*., 2011). Accurate, rapid, sensitive and specific diagnosis of these infections is important in order to determine if the distribution of infections is spreading as well as for better evaluation of management and control programs. Accurate diagnosis using molecular based assays has been achieved by PCR (Hoste *et al*., 1995; Yong *et al*., 2007)

This study demonstrated the challenges that arise in developing molecular diagnostic assays for detection of helminth infections. Optimization of both LAMP and PCR assays developed for genus-specific amplification conducted at different reaction conditions even with altered reagent concentration and volumes yielded no amplification. Various methods of troubleshooting including evaluating different DNA extraction methods and modifying the PCI extraction by addition of different concentrations of 2-mecarptoethanol also proved ineffective. Published PCR primers jhTsp (5'- ttatgtgccacaaatgaaga-3') and NC2 (5′-ttagtttcttttcctccgct-3′) retrieved from Yong *et al*., (2007) were used for troubleshooting the PCR using DNA samples extracted using the different methods mentioned, these also gave no amplification at certified conditions. No amplification was achieved even when the primer set was optimised for annealing temperature. The results achieved with the designed assays may be a direct consequence of improperly designed primers. However, the inability of all primer sets to function for LAMP and PCR as well as published primers suggests problems with the extraction of DNA. This can be attributed to ineffective disruption of worms or the presence of DNases that inhibit amplification.

According to previous studies, it is important that mechanical disruption of worms be followed by cold treatment either by placing tube containing ruptured worms on ice or in the freezer (Karajeh *et al*., 2010), as DNA is rapidly digested by the DNases that

are released on disruption at room temperature. According to Al-Soud and Radstrom (2000), diagnostic PCR is limited in part by the presence of inhibitory substances in complex biological samples, which may interfere with the cell lysis step, inactivate the thermostable DNA polymerase and/or interfere with nucleic acids. The presence of large amounts of DNases in the extracted DNA could explain the inhibition that was observed with both PCR and LAMP in this study. In a previous study investigating the effects of amplification facilitators on diagnostic PCR, Al-Soud and Radstrom (2000) found that using the right combination of thermostable DNA polymerase and amplification facilitators enhances the efficiency of diagnostic PCR. The results of that study indicated BSA, which was also used in this study as one of the most effective amplification facilitators. However, those results were not reflected in the current study. A possible explanation could be that the used batch of BSA was no longer effective or may have been affected by freeze-thaw cycles due to frequent power outages experienced throughout the year. Another possibility could take us back to poor DNA quality.

In conclusion the results of this study suggest the need for evaluation of proper pre-extraction handling of worm tissues samples, mechanical disruption and deactivation of DNases found in ruptured worms. The optimization of DNA extraction methods to decrease the effects of inhibitors in the DNA is also proposed. A previous study has reported a safe and rapid method for isolation of high molecular weight DNA using a common laundry detergent (Bahl and Pfenninger, 1996), a method worth looking into in the future.

CHAPTER 4
DEVELOPMENT OF LAMP AND PCR ASSAYS FOR DETECTION OF *FASCIOLA* SPECIES

4.1 INTRODUCTION

4.1.1 Introduction and classification of *Fasciola* species

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Trematoda
Order: Echinostomida
Family: Fasciolidae
Genus: *Fasciola*
Species: *Fasciola hepatica, Fasciola gigantica*

Members of the genus *Fasciola* are digenean trematodes of the phylum Platyhelminthes. *Fasciola hepatica*, commonly known as the common liver fluke or the sheep liver fluke, and *F. gigantica* commonly known as the giant fluke are parasitic flatworms. The parasites cause disease known as fasciolosis (or fascioliasis) in domestic animals (cattle, sheep, goats, donkeys), wild mammals (rabbits, beavers, deers, water buffalo and camels) and humans (Aldemir, 2006; Cucher, 2006; Aksoy *et al*., 2006; Ahmadi and Meshkehkar, 2010; Alasaad, 2011). Humans in this case are accidental hosts infected through ingestion of raw or undercooked infected meat (Aksoy *et al*., 2006) as well as aquatic plants that are infested with larvae and drinking water that contains these larvae. The flukes have overlapping distributions in some parts of the world especially in Asia and Africa. In particular, *F. hepatica* occurs in temperate zones, and in cooler areas of high altitude in the tropics and subtropics, while *F. gigantica* usually occurs in tropical zones (McGarry *et al*., 2007; Alasaad, 2011). The two are more prevalent in areas with a high stock level of cattle and sheep, where they cause major economic losses through condemnation of infected organs at abattoirs as well as reduced production of meat and milk products (Aldemir, 2006; McGarry *et al*., 2007; Ahmadi and Meshkehkar, 2010; Alasaad, 2011).

4.1.2 Life cycle of *Fasciola* spp.

Parasitic eggs in the faecal material of a primary host (mammals) hatch and release motile miracidia. This takes place at optimum temperature of between 22 - 26°C. Miracidia have only three hours to find a suitable intermediate host before they die. Intermediate hosts are aquatic snails in which sporocytes, radical stages and cercaria develop. These are released from the host shortly after and attach themselves to plants where they encyst and turn into metacercaria. The metacercaria are ingested by herbivorous animals that act as the final hosts. These metacercaria excyst in the small intestines as a result of low pH concentrations and soon find their way through the intestinal wall and peritoneum and finally into the liver capsule (Aksoy *et al*., 2006). The immature flukes migrate through the parenchyma (a process that may take 6-8 weeks), make their way to the small bile ducts and eventually to the larger bile ducts (Aldemir, 2006). Here the parasite matures into an adult fluke and begins to produce eggs (**Fig 12**). Up to 20 000 eggs per fluke per day may be produced in the host resulting in infestations of epidemic proportions.

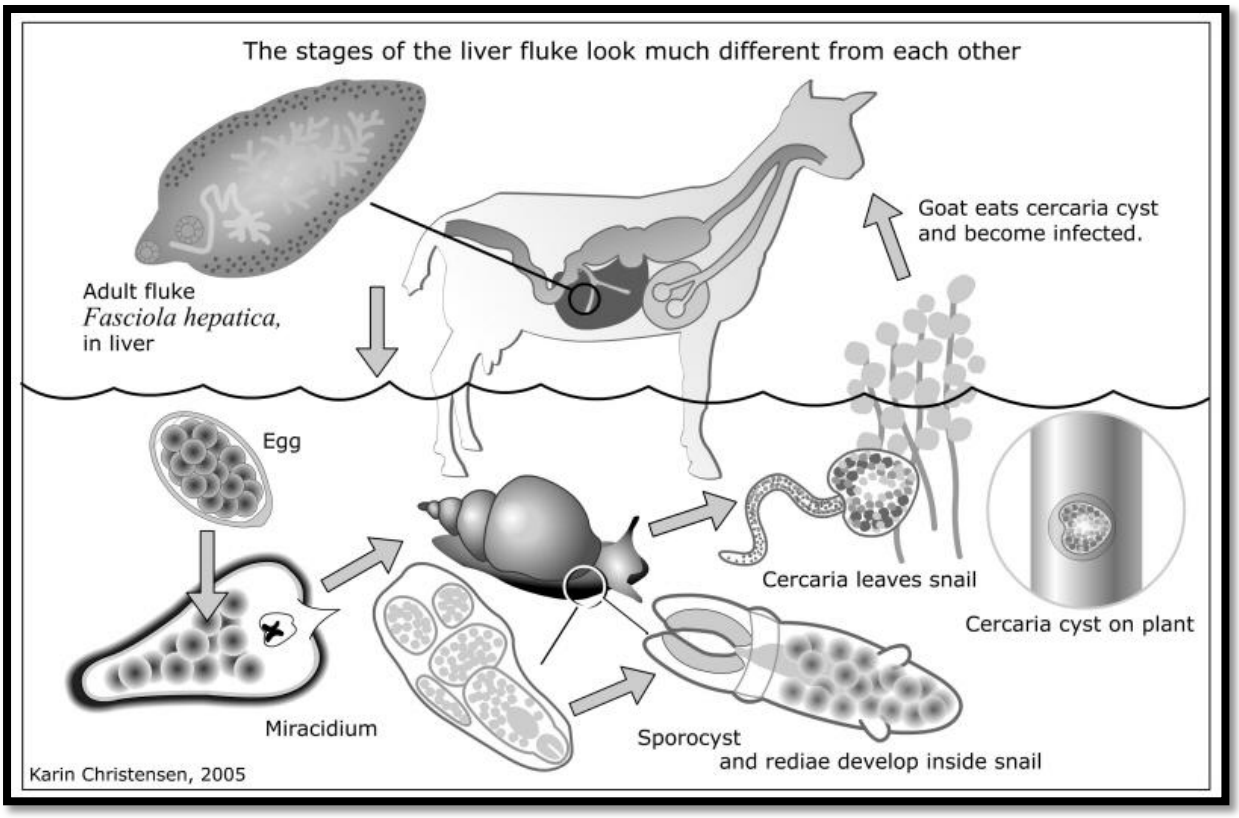


Fig 12: Life-cycle of *Fasciola* spp. This image was extracted from http://www.goatbiology.com/parasites.html

In South Africa, the most common intermediate hosts are members of the genus *Lymnaea* (Aldemir, 2006), commonly known as pond snails that includes the following species: *L. trunculata* (*F. hepatica*), *L. natalenis* (*F.gigantica*) and *L. columella* (*F. hepatica* and *F.gigantica*).

4.1.3 Diagnosis of *Fasciola* spp. infections

Diagnosis of fasciolosis in ruminants is primarily based on the observation of clinical symptoms such as weight loss, anemia, hypoproteinaemia (low blood protein levels) with the inclusion of sudden death and decreased production of wool. Diagnosis has also been achieved through the use of microscopic techniques, which allows the observation of the helminth eggs in faecal samples of both ruminants and humans (Weiss, 1995). A number of immunological techniques have also been developed and used for detection of antibodies developed in the host against infections. These techniques include ELISA and Western Blot methods. However, examination of faecal samples for human infection may lead to a misdiagnosis as the eggs in the stool could be a result of ingestion of egg-containing liver. Diagnosis based on identification of faecal eggs is only possible in late infection between 8 - 12 weeks after initial infection. On the other hand, the use of immunoassays guarantees results within 2 - 4 weeks after initial infection.

Although immunoassay technology seems to produce results more rapidly than microscopy, molecular techniques work better than both methods. Molecular techniques such as PCR, qPCR and LAMP are rapid, sensitive and specific and therefore detect infection at its early stages in just less than 4 hours. Alasaad *et al*., (2011) reported the first qPCR assay for identification of *Fasciola* spp., which proved successful in identification of both taxonomically valid species of *Fasciola*, *F. hepatica* and *F. gigantica*, as well as the newly discovered intermediate form. The assay like many others reported so far was validated on adult flukes alone and is therefore limited in sensitivity as per the goals of this study. Ai *et al*., (2010a) developed a specific PCR-based assay for identification of *Fasciola* species. The assay proved both specific and sensitive allowing for species-specific detection with as little as ~1 ng/µl of genomic DNA. The success of the assay is attributed to the use of species-specific primers that were designed using DNA from the nuclear ribosomal second internal transcribed spacer gene (ITS2), which according to Schnieder *et al*., (1999) is a useful tool for making species delineations. The

developed PCR assay is an essential diagnostic tool that could prove epidemiologically significant and is representative of the assays that are in demand. The qPCR assay, however, proved more sensitive than the traditional PCR assay as shown by the difference in detection limits of both assays (~1 ng/µl and ~1 pg/µl for traditional and qPCR respectively).

A novel diagnostic LAMP assay has been reported for species-specific differentiation of *Fasciola* species (Ai *et al*., 2010a). The assay, validated on eggs, larvae and adults of *F. hepatica* and *F. gigantica*, can essentially detect all three life stages of the two parasites. This is a desirable feature for all species-specific molecular diagnostic tools yet to be developed. This breakthrough should significantly aid in detection and control of human and animal fascioliasis thereby reducing the negative impact on human health and the economy.

4.1.4 Objectives

- To develop a LAMP assay for detection of *Fasciola* species infections in cattle and sheep.
- To develop a PCR assay for detection of *Fasciola* species infections in cattle and sheep.
- To apply the newly developed LAMP and PCR assays in determining the prevalence of *Fasciola* spp.

4.2 MATERIALS AND METHODS

4.2.1 Helminth samples used for positive and negative control purposes

For the purpose of this study, the adult worms (*Fasciola hepatica*, *F. gigantica*, *Haemonchus contortus*, *H. placei*, *Trichuris spp.*, *Oesophagostomum radiatum*, *Trichostrongylus* spp., *Schistosoma mattheei*, *Cooperia* spp., *Avitellina* spp., and *Paramphystomum* spp.), were obtained from the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, South Africa. DNA was extracted from these worms by salting out as described below.

4.2.2 Faecal sample collection and storage

Fresh faecal samples were collected from cattle, sheep and goats in four South African Provinces. The samples were processed and preserved as stated in **Chapter 2 Section 2.2.2**.

4.2.3 Methods used for DNA extraction and troubleshooting

The different methods used for DNA extraction and troubleshooting are outlined in **Chapter 2 Section 2.2.4**.

4.2.4 Amplification methods for detection of *Fasciola* spp. DNA

i. PCR

The PCR primers including, FFhepF 5'-gcggccaaatatgagtca-3', FhepR 5'-ctggagattccggttaccaa-3', FgigF 5'-gttcaggtgacaagccaa-3', and FgigR 5'-atcacaccgtgaagcaga-3', for specific amplification of the RAPD sequences of *F. hepatica* and *F. gigantica* respectively, and published by McGarry *et al*., (2007) were purchased and used for PCR reactions to confirm the identity of the adult worms that were to be used as positive controls. PCR was performed using 12.5 µl Dream *Taq* mix, 10 pmol of each forward and reverse primer, 5.5 µl ddH2O and 5.0 µl template DNA. The reactions were setup to amplify the gene at an initial denaturation of 94°C for 3 min followed by 30 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min.

For optimising the developed assays, all PCRs were setup to the volume of 25 µl. Initial attempts at species-specific amplification was evaluated with 2.5 µl PCR buffer (QIAGEN), 0.5 µl (5mM) dNTPs, 10 pmol primer mix (made up of 20 µl of each forward and reverse primer stock and 60 µl ddH_2O), 0.1 µl of HotStar *Taq* DNA

polymerase, 1.0 µl of BSA, 11.9 µl of ddH_2O and 3.0 µl of template DNA. Reactions were incubated in a MultiGene OptiMax Thermal Cycler (Life Technologies, USA). Amplification was done under the following conditions PCR conditions: 95°C for 15 min followed by 35 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 10 min. Thereafter, genus-specific amplification was also evaluated using 12.5 µl of Dream *Taq* Green Mix, 10 pmol of each forward and reverse primer, 1.0 µl of BSA, 4.5 of µl ddH_2O and 5.0 µl of template DNA at an initial denaturation 95°C for 3 min followed by 35 cycles of at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final extension at 72°C for 7 min. Different types of PCR master-mixes, buffers and DNA polymerases were used for troubleshooting (**Table 3 Chapter 2**) for both species-specific and genus-specific primers. The reaction conditions were altered to suite each reaction depending on the denaturation temperature required for activating the enzyme in each mastermix. Reagent concentrations and volumes were also altered a number of times and several annealing temperatures ranging from 50°C - 60°C were tried and tested together with different incubation periods for each step.

ii. LAMP

Published LAMP primers for detection of *F. hepatica* and *F. gigantica* (**Table 5**) were also purchased and tested with DNA extracted from adult worms of the two species. The 25 µl reaction mixture consisted of 12.5 µl LAMP Buffer, 1 µl *Bst* DNA polymerase, 1 µl fluorescent detection reagent (FD), primer mix (0.2 mM of each F3 and B3 primer, and 1.6 mM of each FIP and BIP and 0.8 mM of each LF and LB primer), 7.2 µl ddH_2O and 2.0 µl template DNA. LAMP conditions used to achieve desired amplification were as follows; 62°C for 60 min followed by 80°C for 5 min in order to inactivate the enzyme and final incubation at 4°C. The F3 and B3 primers were also used in PCR reactions to amplify target DNA using PCR reagents and conditions stipulated above (**Section 4.2.4 i**).

All reactions for assay optimization were 25 µl volumes and consisted of 12.5 µl LAMP buffer, 1 µl *Bst* DNA polymerase, 1 µl fluorescent detection reagent (FD), primer mix (0.2 mM of each F3 and B3 primer; 1.6 mM of each FIP and BIP primer as well as 0.8 mM of each LF and LB primer), 2.5 µl template DNA and enough ddH_2O to make a total volume of 25 µl.

Table 5: Published LAMP primers for species-specific detection of *Fasciola* spp. (Ai *et al*., 2010b)

Primer	*Fasciola hepatica*	*Fasciola gigantica*
F3	5′-cattaccgactcagcttgca-3′	5′-cactgcgagacactgagtc-3′
B3	5′-accaaacgttcggttaaggt-3′	5′-gcacacaagtcagtcaagca-3′
FIP	5′-gccgaatcaaccagccctgaaa atgacggtccggtataggtc-3′	5′-gcacacaagtcagtcaagca atgatttgagggcacgacc-3′
BIP	5′-agcggattccaacttccatggc acgcgacgctcatgagat-3′	5′-tcgttgggtagtgaacatgggg acacaaatggacgcagaca-3′
LF	5′-gatggcgctggagcgtcgga-3′	5′-tggggtggatttcctcgc-3′
LB	5′-caccgtcctgctgtctgg-3′	5′-gtctacaaacgatttattgc-3′

4.2.5 Product visualization

The products of each DNA extraction, PCR and LAMP were analysed by agarose gel electrophoresis as detailed in **Chapter 2 Section 2.2.4**.

4.2.6 Development of LAMP and PCR assays for detection of *Fasciola* spp.

i. Primer design

Two primer sets targeting the Internal transcribed spacer 2 (ITS2) for specific amplification of *F. hepatica* and *F. gigantica* were designed using the nucleotide sequences with accession numbers JF496716 and HQ197358 respectively. The primers were downloaded from the NCBI GenBank and then uploaded onto LAMP primer design software, PrimerExplorer V4. The software generated 5 sets of primers from which one set that annealed closest to the target gene was chosen (**Table 6**). The primer set F3, B3 for each species were used for specific amplification of *F. hepatica* and *F. gigantica*. A second set of PCR primers (FasF1: 5’-cgtctctcggggcgcttgtc-3’ and FasR1: 5’-gccactgcacctcaccacgg-3’) for genus-specific amplification was designed using Primer Blast, NCBI.

Table 6: Designed LAMP primers for specific detection of *F. hepatica* and *F. gigantica*

Primer	*F. gigantica*	*F. hepatica*
F3	5'-gaccgtcatgtcatgcgata-3'	5'-tccaagccaggagaacgg-3'
B3	5'-ccgtagcccaaatctcctct-3'	5'-atcagtccaacccgggata-3'
FIP	5'-gtaggcaccccatcagtgatcgaattt gcggacggctatgc-3'	5'-gttcatagggcgggagcaggactgccacga ttggtagtg-3'
BIP	5'-actccgatggtatgcttgcgtctcatg gcagtacaacccgt-3'	5'-agtggtactgaatggcttgccagacgtacg tgcagtccag-3'
LF	5'-gctgtgacctcaatgagcc-3'	5'-gcccaaatctcctctttaagcctag-3'
LB	5'-gcgcttgtccaagccagga-3'	5'-ttctttgccattgccctcgc-3'

ii. Optimization of the assay

LAMP

A number of LAMP reactions were setup and ran in a Loopamp Turbidimeter (Teramecs, Japan), at various conditions, in order to find suitable conditions for amplification. Temperatures ranging from 60°C to 65°C and incubation time of 60 min - 120 min were explored.

PCR

PCR system was set up to amplify the internal transcribed spacer 2 (ITS2) gene of both *F. hepatica* and *F. gigantica* to be able to determine optimal conditions for primer annealing temperature. The reactions were set up as outlined in **Section 4.2.4 i.** above.

4.3 RESULTS

4.3.1 Confirmation of control DNA samples by LAMP and PCR

In order to confirm the identity of control tissue samples published PCR and LAMP primers were used to amplify DNA extracted from the two *Fasciola* species. No amplification was obtained with the PCR primers retrieved from McGarry *et al*., (2007) designed to target the RAPD sequence. However, positive PCR identification of the parasite DNA was achieved using the F3, B3 primers initially designed for use in a LAMP reaction published by Ai *et al*., (2010b). The primers targeted the ribosomal intergenic spacer of the nuclear genome.

For LAMP assay the published primers (**Table 5**) used for amplification of *F. hepatica* DNA did not give any positive results with either target or non-target DNA. On the other hand published *F. gigantica* primers were cross reactive, amplifying both *F. gigantica* and *F. hepatica* DNA.

4.3.2 Optimisation of LAMP assay

Testing of the developed LAMP assays for species-specific detection of *Fasciola* species yielded negative results initially. The primer set designed for specific amplification of *F. hepatica* DNA did not yield any positive bands when tested at various annealing temperatures. Negative results were obtained even when the incubation period was increased from 60 min to 120 min. The assay developed for specific detection of *F. gigantica* DNA gave what appeared to be non-specific amplification (**Fig 13**). Therefore, various troubleshooting mechanisms were tried, however, the non-specific DNA was still amplified, similarly so was the negative control with ddH_2O.

4.3.3 Optimisation of PCR assay

At first, PCR for species-specific detection of both *F. hepatica* and *F. gigantica* DNA yielded no amplification product (**Fig 14**). A second attempt using the specific *F. gigantica* primers yielded false positive PCR product with the negative control containing ddH_2O. This was regarded as contamination as subsequent trials gave no amplification at all. Various PCR conditions were tested together with DNA samples extracted using different methods but these yielded negative results in both cases.

Another PCR assay was developed using the genus-specific primers tested at various conditions and using DNA extracted by PCI, salting out and by the QIAamp Mini DNA Kit. The results were the same as those achieved with species-specific primers as there was no amplification product in all the samples tested (**Fig 15**).

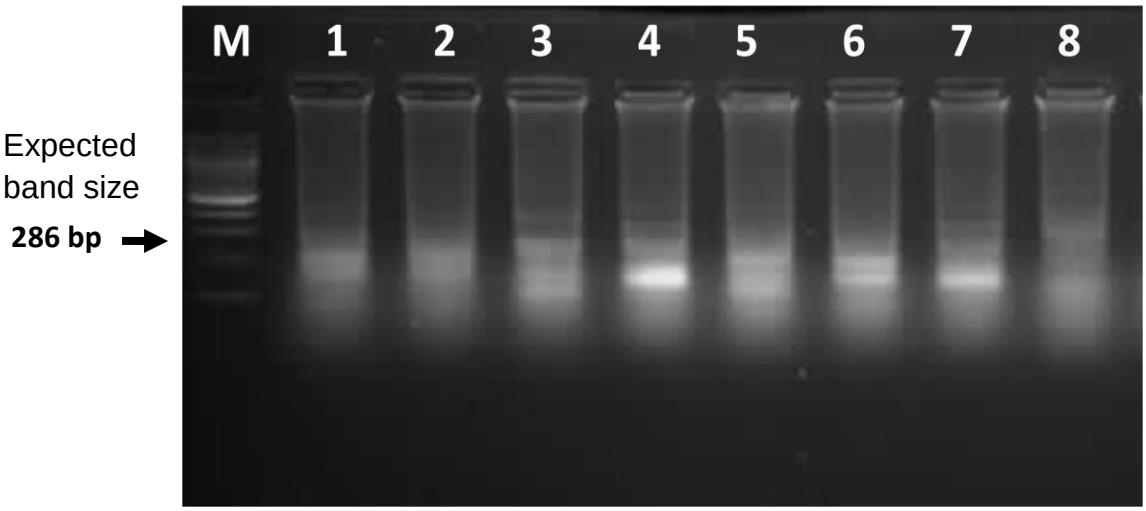


Fig 13: Specific detection of *F.gigantica* DNA after amplification by LAMP assay and agarose gel electrophoresis (1%). The gel shows non-specific LAMP product. (M) 100 bp Molecular marker, (1) ddH_2O, (2) *F. gigantica*, (3) *F. hepatica*, (4) *H. contortus*, (5) *H. placei*, (6) *Paramphistomum* sp., (7) *Schistosoma mattheei*, (8) *Avitellina* spp.

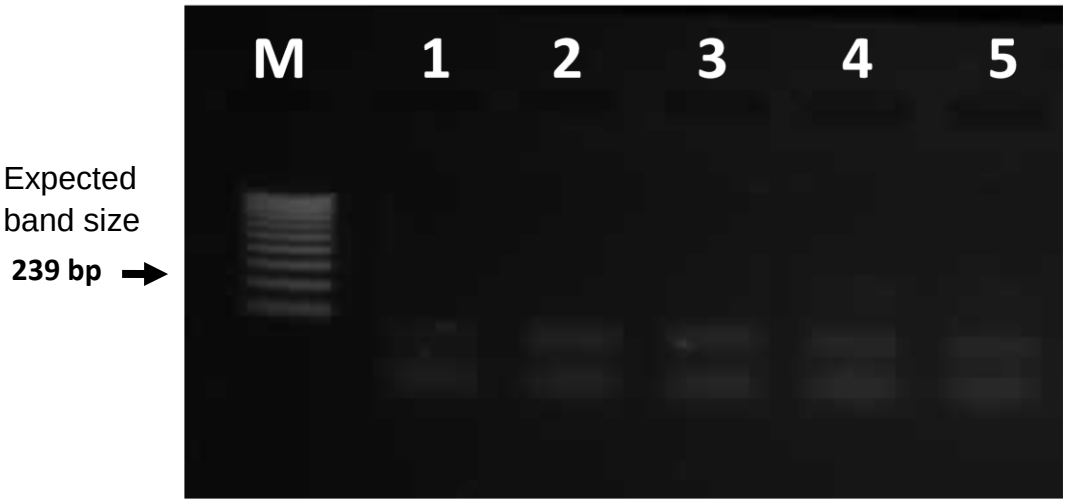


Fig 14: Specific detection of *F. hepatica* DNA extracted using different extraction methods, amplified by PCR assay and subjected to electrophoresis on 1% agarose gel. (M) 100 bp Molecular marker, (1) ddH_2O, (2) PCI extraction, (3) Salting out, (4) Kit extraction, (5) PCI coupled with 2-Mercaptoethanol

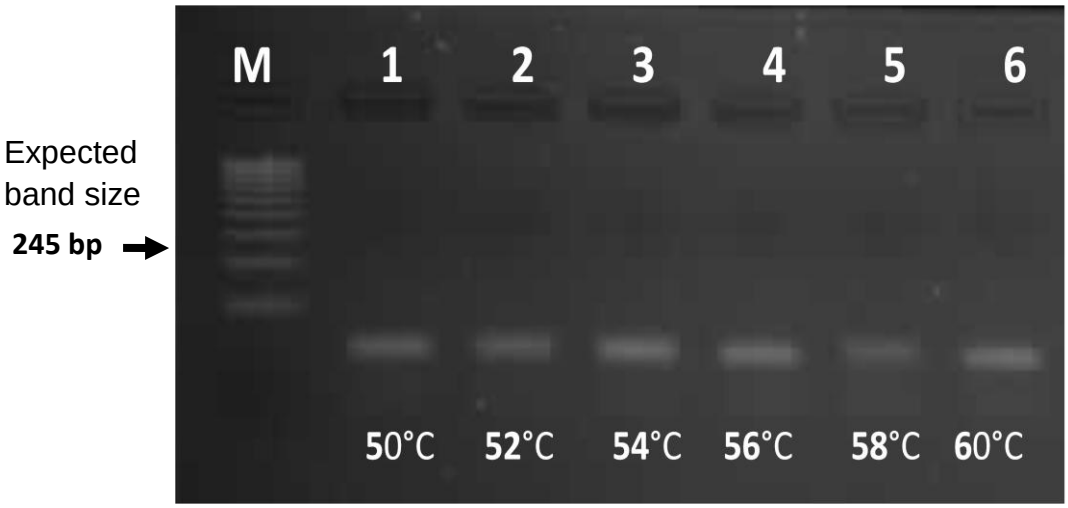


Fig 15: Genus-specific detection of *Fasciola* spp. DNA after amplification at different annealing temperatures and separation of PCR product on 1% agarose gel. The gel shows non-specific PCR product at all tested annealing temperatures.

4.4 DISCUSSION AND CONCLUSIONS

Flatworms of the genus *Fasciola* are parasites of economic significance infecting livestock and have been reported as human health hazard globally (Ali *et al*., 2008; Ai *et al*, 2010a; Ai *et al*, 2010b). Due to the devastating effects of the infection on livestock production accurate and early detection is crucial to their management and control (Ai *et al*, 2010a; Ai *et al*, 2010b). Traditional methods of diagnosis including identification and differentiation based on morphological characters as well as microscopic examination of faecal eggs is often time consuming, labour intensive and subject to errors due to subtle morphological differences that may be missed during identification (Ai *et al*, 2010a; Ai *et al*, 2010b). Identification may also be biased based on the experience and knowledge of the technical expert. Recent developments in DNA based technology have enhanced identification and differentiation of *Fasciola* spp (Cucher *et al*., 2006; Magalhães *et al*., 2008; Ali *et al*., 2008; Ai *et al*., 2010a; Ai *et al*., 2010b).

The aim of this study was to develop LAMP and PCR assays for detection of *Fasciola* species infections in livestock. The assays were designed to target the ITS2 sequence of *Fasciola* spp. as this has been identified as a reliable species-specific marker for identification and differentiation of *Fasciola* spp. (Ali *et al*., 2008; Ai *et al*., 2010a) Initially primers for species-specific amplification by LAMP were designed targeting the ITS2 gene. From these, the outer primers (F3 and B3) were used for PCR amplification. The assays yielded negative results on several occasions. The LAMP assay for specific detection of *F. gigantica* DNA yielded non-specific amplification product. According to Eiken Chemical Co., Ltd (2005), non-specific amplification is often a result of either reagent contamination or the formation and amplification of primer dimmer. In order to eliminate contamination, stocks solutions of all the reagents were discarded and new ones were used. Reagent volumes and concentrations were altered. The quantity of template DNA used was increased or decreased as was determined best and annealing temperature together with amplification times were altered a number of times to optimise. However, none of these changes made any difference as non-specific amplicons were still detected on agarose gel electrophoresis and by Loopamp real-time turbidimeter. These results were consistent with those of Stridiron *et al*., (2009) who also obtained false positives with LAMP even after optimization. This therefore suggests that

contamination was not the source of false positives in this study and a closer look into primer design needs to be taken since according to Ai *et al*., (2010a), this is an important step in assay development.

A second primer set was designed for genus-specific detection by PCR with which no positive amplification product was obtained even after optimizing. At this point, a more likely explanation for the ineffective assays was thought to be poor quality of DNA or the lack thereof. However, various methods of DNA extraction were evaluated in order to maximise the DNA yield. The addition of 2-mecarptoethanol following mechanical disruption did not produce desired results with modified DNA extraction. According to previous studies it is important that mechanical disruption of worms be followed by cold treatment either by placing tube containing ruptured worms on ice or in the freezer (Karajeh *et al*., 2010), as DNA is rapidly digested by the DNases that are released on disruption at room temperature. The presence of large amounts of DNases in the extracted DNA may explain the inhibition that was observed after both PCR and LAMP assays.

PCR inhibition may be attributed to the presence of inhibitory substances in complex biological samples. This sample matrix may interfere with the cell lysis step and even inactivate the thermostable DNA polymerase resulting in interference with nucleic acids (Al-Soud and Radstrom, 2000; Al-Soud *et al*., 2005). Adult worms of the genus *Fasciola* inhabit the bile ducts of their hosts. Bile has been previously reported to contain PCR inhibitory components (Al-Soud *et al*., 2005). Residues of these PCR inhibitory components may have contaminated the adult worms thereby contributing to the inhibitory effect of the extracted DNA on the amplification of PCR products.

The efficiency of PCR can be enhanced by the use of a thermostable DNA polymerase and amplification facilitators as demonstrated in previous studies (Al-Soud and Radstrom, 2000; Al-Soud *et al*., 2005). In this study, however, the use of BSA, which was reported to be one of the most effective amplification facilitators in conjunction with *Taq* DNA polymerase or the mastermixes containing *Taq*, which is thermostable did not produce the desired results. This outcome could have resulted from the use of an old and possibly ineffective batch of BSA. The BSA could have also have had little effect in facilitating amplification because of the poor quality of template DNA used.

The results of this study therefore suggest evaluation of proper pre-extraction handling of worm tissue samples, mechanical disruption and deactivation of DNases found in ruptured worms and optimization of DNA extraction methods to decrease the effects of inhibitors in the DNA.

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Poor muscle and skeletal development, reduced fertility, reduced production of meat, milk and wool as well as high rate mortalities are some of the effects of helminth infections that threaten global livestock production and economic growth (Schnieder *et al*., 1999; Tsotetsi and Mbati, 2003; Aldemir, 2006; Hale, 2006; Bott *et al*., 2009; Jackson *et al*., 2009; Ahmadi and Meshkehkar, 2010; Ahmed, 2010; Maphosa *et al*., 2010). Developing simple, rapid and accurate detection systems is of great importance for the diagnosis of disease, and identifying pathogens at an early stage is significant for disease control and prevention (Karajeh *et al*., 2010).

The aim of this study was to develop molecular diagnostic assays for detection of *H. contortus*, *Fasciola* spp. and *Trichostrongylus* spp. infections in cattle, sheep and goats. The basis for development of molecular diagnostic assays is not to replace but to supplement existing methods of diagnosis with more rapid and accurate ones. Although molecular diagnostic tools have many attributes that render them superior to traditional diagnosis using microscopy, they do, however, possess some disadvantages. One of the shortcomings of screening DNA extracted directly from faecal samples using molecular tools is the inability to distinguish between patent and non-patent sources of DNA (Sweeny *et al*., 2011; Sweeny *et al*., 2012). Hence, traditional methods of diagnosis are still necessary. Moreover, even though LAMP through the use of a Loopamp real-time turbidimeter is able to quantify infections, PCR as a diagnostic technique is not quantitative; therefore worm egg counts are necessary in order to quantify the magnitude of infections (Sweeny *et al*., 2011).

A number of molecular based assays have been developed for detection of helminth infections in livestock especially sheep. However, most of them were conducted using genomic DNA extracted from eggs, which have been separated from faeces using sodium nitrate or by column purification (Bott *et al*., 2009; Roeber *et al*., 2011). Similar to the Sweeny *et al*., (2011) method, the assays developed in this study were to be validated on genomic DNA extracted directly from unprocessed faecal samples. However, this proved rather difficult as the primers used may have been ineffective. Furthermore, the ineffective methods of DNA extraction and the presence of inhibitors in DNA extracted directly from faecal samples may also contribute to

failures of the developed LAMP and PCR assays. This would explain why a number of studies would use column purified eggs to limit the effect of faecal inhibitors on PCR.

In this study, an optimised LAMP assay detected *H. contortus* DNA. However, optimisation of other designed assays failed to work even after several attempts, an observation initially attributed to ineffective primers. Non-specific amplification product was observed with the designed LAMP assay for specific detection of *F. gigantica* indicating a problem with primer design. However, the primers were not the only cause for concern in this study as subsequent trials with the once functional, optimized *H. contortus* assay were unsuccessful, suggesting either faulty primer manufacturing by the primer manufacturing company or ineffective methods of DNA extraction.

Various methods of DNA extraction evaluated in the study did not yield positive results. This could be attributed to the presence of PCR inhibitors that were not either removed or inactivated during DNA extraction. According to literature, mechanical disruption of worms during DNA extraction should be followed by immediate cold treatment by placing tubes containing ruptured worms on ice or in the freezer to prevent degradation of DNA by DNases that are released from the worms (Karajeh *et al*., 2010). A more risk-free method of disruption would be shock-freezing the worms in liquid nitrogen and crushing them while they are still frozen (Rufener *et al*., 2009). This can then be followed by standard PCI extraction. According to Harmon *et al*., (2007), the effect of faecal inhibitors can be minimised by treating the eggs with phytase before extraction or by the addition of BSA in the reaction tubes. On the other hand, the effects of PCR inhibitors can be reduced by using a thermostable DNA polymerase in conjunction with amplification facilitators such as BSA, betaine, proteinase inhibitor and single-stranded DNA binding T4 gene 32 protein (gp32). The right combination of amplification facilitators has also be proved to enhance PCR yield (Al-Soud and Radstrom, 2000).

In this study, faecal egg counts were only conducted on a few samples for sensitivity purposes. However, the importance of using the egg count techniques and molecular diagnosis is acknowledged, in order to confirm the reliability of the output data as well as to compare the specificity and sensitivity of assays. Or in this case

the egg counts would have been useful in determining the prevalence of the target pathogens as challenges were encountered with developing and/or validating molecular assays.

In conclusion, this study has developed specific and sensitive PCR and LAMP assays for detection of *H. contortus* infections. However, the assays were not tested further with field samples due to suspected poor quality of genomic DNA and the presence of faecal inhibitors in the PCR mixture. Unfortunately, this study was failed to develop PCR and LAMP assays for detection of *Trichostrongylus* and *Fasciola* spp. infections. Perhaps, ineffective extraction of genomic DNA and ineffective primers may have caused this failure. Evaluation of more accurate methods of mechanical disruption, DNA extraction, primer design and the use of amplification facilitators have the potential to yield desired results and should therefore be taken into account for successful development and validation of molecular diagnostic tool for detection of helminth infections.

In light of the challenges that were encountered in this study regarding development and/or validation of molecular diagnostic assays for detection of helminth infections in livestock, it is recommended that extraction of DNA from adult worms and from faecal samples be closely evaluated. DNA extraction from adult helminth worms may be problematic as was the case in this study. This is due to the presence of high levels of DNases and this can be overcome by freezing the mechanically disrupted worms as soon as possible after disruption. Harmon *et al*., (2007) suggest the use of phytase during DNA extraction in order to reduce the effects of faecal inhibitors. Another recommendation would be spiking tissue samples with tissue from other pathogens, which will work as a control. DNA can then be extracted from the mixed tissues followed by PCR to be able to amplify the foreign DNA, which would signal the presence or absence of inhibitors. The inclusion of these changes in DNA extraction methods and proper primer design may be a sure way to achieving the desired results. Alternatively, extracted DNA can be spiked with foreign DNA and then amplified by PCR using primers designed to amplify the foreign DNA. This ensures that any inhibitors that may have remained after DNA extraction are determined. The use of a detergent in PCR assays is advisable to increase the sensitivity of the designed assays.

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