DEVELOPMENT OF MOLECULAR AND SEROLOGICAL ASSAYS FOR DIAGNOSIS OF BOVINE (*TAENIA SAGINATA*) AND PORCINE (*TAENIA SOLIUM*) CYSTICERCOSIS IN SOUTH AFRICA

By

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DECLARATION

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

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RESEARCH OUTPUTS

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ABBREVIATIONS AND SYMBOLS

>	**Greater than**
<	**Less than**
µl	**Microliter**
µM	**Micromolar**
mM	**Millimolar**
ml	**Millilitre**
nm	**Nanometer**
ng/µl	**Nanograms per microliter**
pg	**Picograms**
A_{260}/A_{280}	**Ratio of absorbance monitored at 260nm and 280nm**
ATL	**Tissue lysis buffer**
AE	**Elution buffer**
AW	**Wash buffer**
Bp	**Base pair**
***Cox*1**	**Cytichrome C oxidase subunit1**
Cp	**Crossing point**
Ct	**Threshold cycle**
°C	**Degree Celsius**
DNA	**Deoxyribonucleic acid**
DNTPs	**Deoxy-nucleotide triphosphate**
EDTA	**Ethylenediaminetetraacetic acid**

E/S	**Excretory/secretory**
EIA	**Enzyme immunoassay**
ELISA	**Enzyme-linked immunosorbent assay**
FAM	**Reporter 6-carboxyfluorescein**
IVM	**Ivermectin**
ITS-1	**Internal transcribed spacer 1**
LAMP	**Loop-mediated isothermal amplification**
kDa	**kilo Daltons**
$MgCl_2$	**Magnesium chloride**
OIE	**Office International des Epizooties (World Organisation for Animal Health)**
OD	**Optical density**
OVI	**Onderstepoort Veterinary Institute**
NaCl	**Sodium chloride**
NaOAc	**Sodium acetate**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
RAPD	**Random amplified polymorphic DNA**
RNA	**Ribonucleic acid**
Rn	**Normalized reporter**
rpm	**Revolutions per minute**
SCAR	**Sequence characterized amplified region**
SDS	**Sodium dodecyl sulphate**

Taq	***Thermus aquaticus***
TAE	**Tris acetate EDTA**
TAMRA	**Quencher tetramethylrhodamine**
Tris-HCl	**Tris-(hydroxymethyl)-aminomethane-hydrochloric acid**
U	**Unit**
UV	**Ultraviolet light**

ABSTRACT

Cysticercosis is an infection of cattle and pigs caused by metacestodes of human tapeworms, *Taenia saginata,* and *T. solium*. Currently, meat inspection is the standard method used for diagnosis of cysticercosis and has proven to be a less sensitive and subjective. The current study was therefore aimed at improving and developing specific and sensitive molecular assays for detection of *T. saginata* and *T. solium* infections in cattle and pigs respectively. Furthermore, this study also sought to assess the potential of antigen detection ELISA for diagnosis of cystircercosis in South Africa. The currently available conventional PCR assays respectively targeting HDP2 and *cox*1 genes were optimised for use under South African conditions, whilst real-time PCRs (qPCR) targeting the *cox*1 gene of both *T. saginata* and *T. solium* were newly developed. The HDP2 gene PCR assay was successfully optimised, however no positive results were obtained in the field samples. However, the assay targeting the *cox*1 gene yielded positive results in both the control and field samples. All cyst samples collected from bovine and porcine carcasses tested positive, but only bovine blood samples tested positive with a prevalence of 94% (577/614) with no positive results obtained in the porcine blood samples. The *T. saginata* and *T. solium* qPCR assays were successfully developed with respective detection limits of 0.0013 ng/µl and 0.0034 ng/µl. Both assays only detected target species, thus showing good specificity. The assays respectively confirmed 63% (45/71) and 100% (2/2) *T. saginata* and *T. solium* cysticerci respectively, further detected 75% (458/614) of *T. saginata* and 33% (76/233) of *T. solium* infections in bovine and porcine blood samples. Furthermore, a bovine cysticercosis sero-prevalence of 5.6% (18/320) was obtained through HP10 AgELISA, although meat inspection recorded 0% prevalence. The study also showed that MoAb (HP10) antigen detecting ELISA is more sensitive than meat inspection in the diagnosis of taeniid infection in cattle. Both conventional and real-time PCR assays targeting the *cox*1 gene proved that they can be used as confirmatory tools for meat inspection results made at abattoirs and have the potential to be used as pre-mortem diagnostic tools for detection of *T. solium* and *T. saginata* infections in cattle and pigs. Further validation of the developed qPCR assays using known taeniid species positive blood samples is recommended.

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND AND LITERATURE REVIEW

Cysticercosis is a muscular infection caused by metacestodes of *Taenia saginata and T. solium*, the two taeniids of greatest economic and medical importance. They cause taeniosis in humans and cysticercosis in cattle and pigs/humans respectively. Taeniosis occurs when humans become infected by eating undercooked meat with metacestodes of these taeniid tapeworms (Gonzalez *et al*. 2006). It is universally distributed in both developing and developed countries (Murrell 2005). Estimates of approximately 50 million cases of such infestation is estimated to occur worldwide with 50,000 people dying from taeniosis (Wanzala *et al*. 2003) and neurocysticercosis (Zirintunda & Ekou 2015) annually.

Animals get infected by grazing contaminated pastures or ingestion of human faeces or drinking water contaminated with faeces containing tapeworm's eggs (Dutra *et al*. 2012; Karshima *et al*. 2013). Cysticerci are found anywhere in the body of the pig, most commonly in the muscle and subcutaneous fat and in the brain (García *et al*. 2003). In cattle, cysticerci are commonly found in the heart and skeletal muscles, and are sometimes found in other sites such as the liver, lung, kidneys and lymph nodes (Scandrett 2007). Live cattle having cysticercosis show no symptoms, however, heavy infestations cause myocarditis or heart failure (Wanzala *et al*. 2003). Both bovine and porcine cysticercosis cause economic losses due to condemned, downgraded carcasses and treatment of carcasses before human consumption (Wanzala *et al*. 2003; Sciutto *et al*. 1998).

Although cattle and pigs act as intermediate hosts, humans can also act as intermediate hosts for *T. solium* by accidentally ingesting this taeniid eggs. They develop the cystic form either through faecal-oral transmission from contaminated materials (Abuseir *et al*. 2007) or autoinfection caused by rupturing of the tapeworm causing eggs to be released in human intestines and thus developing cysticercosis (Ramahefarisoa *et al*. 2010; Garcia *et al*. 2003). In humans, metacestodes of *T. solium* are commonly found in the heart and striated muscles and the subcutaneous tissues (Shore 2001), the eyes, the brain and the central nervous system after ingestion of the *T. solium* eggs (Garcia *et al*. 2003). Taeniid species have different impact on human beings, with *T. solium* being the most important by causing fatal neurocysticercosis.

Taenia infections are common in environments with poor sanitation, extensive livestock husbandry practices, and inadequate meat inspection, management and control policies

(Garedaghi *et al*. 2011) and where inhabitants traditionally eat raw or insufficiently cooked or sun-cured meat (Kumar & Tadesse 2011; Pondja *et al*. 2010). Porcine cysticercosis is emerging as a public health and agricultural problem of concern in lesser developed areas (Rajshekhar *et al*. 2003; Ito *et al*. 2004).

Development of improved sanitation and hygiene practices have a major impact on the occurrence of cysticercosis in developed countries, and also among urban dwellers in developing countries, because of their effect on the transmission of taeniid eggs (Murrel *et al.,* 2005). Public education on the use of latrines and improved standards of human hygiene and avoiding consumption of raw meat are practical measures that can also be practised to prevent transmission of taeniid eggs from infected humans to livestock (Wondimagegnei & Belete 2015; Kumar & Tadesse 2011). Water is regarded as an important factor in the transmission of bovine cysticercosis to a herd and the prevalence and the geographic distribution indicated that a variety of potential risk factors or practices present maintain the cycle of *T. saginata*. Farmers are advised to restrict the access of their cattle to surface drinking water and supply them with fresh water instead. Farmers should be fully supported and informed of the life cycle of *T. saginata* and potential risk factors for cattle to become infected. Risk factor studies carried out in a well-defined cattle population, in a well-defined type of farm, in a more limited area and including a more sensitive and specific diagnosis of bovine cysticercosis, using serology (e.g. Ag-detection ELISA) should be encouraged. (Boone *et al.,* 2007). Proper digestion and sanitisation of sludge is a guarantee of a negligible risk for cattle and human health (Cabaret *et al.,* 2002).

The use of vaccines is an alternative approach for the control of taeniosis and cysticercosis (Flisser & Lightowlers 2001). A number of approaches such as the use of parasite crude extracts (Flisser *et al*. 2004), protein sub-unit vaccines (Morales *et al*. 2008) and DNA vaccination (Guo *et al*. 2007) have been used to developed vaccines against bovine and porcine cysticercosis.

Crude antigens obtained from *T. solium* oncospheres, cysticerci or tapeworms have been used and it was found that living oncospheres and oncospheral antigens are the most effective in providing protection against porcine *T. solium* cysticercosis (Flisser & Lightowlers 2001). The use of recombinant proteins and DNA as vaccines against rodent, ovine and bovine cysticercosis has been used with high degrees of immunity (Harrison *et al*. 1996; Lightowlers *et al*. 1996).

Vaccination with a combination of two antigens, designated TSA-9 and TSA-18, induced up to 99.8% protection against experimental challenge infection with *T. saginata* eggs. The vaccine has the potential to be used on a commercial scale for the control of bovine cysticercosis (Lightowlers *et al*. 1996).

Flisser *et al*. (2004) demonstrated that the TSOL18 and TSOL45-1A, recombinant antigens cloned from the larval oncosphere stage of the *T. solium* parasite induce very high levels of protection against *T. solium* infection in pigs and provide a solid basis on development of a practical vaccine to assist with control and potential eradication of human neurocysticercosis. Immunization of pigs with the TSOL18 recombinant antigen alone induced complete, or near-complete (99.5%), protection against the development of cysticerci while The TSOL45-1A antigen induced a high level of protection (97%) against the challenge infection with *T. solium*. Vaccination with a combination of both TSOL18 and TSOL45-1A induced a 94.7% reduction in the number of cysticerci, with two individual pigs having no cysticerci detected.

Vaccination trials performed by Cai *et al*. (2008) showed that vaccination with the recombinant TSOL18 antigen reduced the number of cysticerci recovered giving a 94% reduction 5% higher than the 89% reduction induced by using cysticercus crude extracts. More recently, TSOL18 has been proven to be highly effective against naturally acquired infection with *T. solium* in pigs. Application of TSOL18 together with a single treatment of pigs with oxfendazole achieved complete elimination of transmission of the parasite (Lightowlers 2010; Assana *et al*. (2010).

The TSOL16 antigen is a third *T. solium* antigen type isolated from *T. solium* and that has been cloned from oncospheres and the encoding gene has been characterized and has been able to confer high levels of protection against challenge infection with *T. solium* (Gauci *et al*. 2012). Pigs vaccinated with TSOL16 showed a significant reduction in the number of cysticerci and provide 99.8% protection and 97.9% protection was observed in pigs vaccinated with TSOL45-1A antigen. A very low protection of 18.8% was observed in a group of pigs vaccinated with TSOL45-1B. The results of the vaccine trial in which pigs were immunized with the TSOL16 recombinant antigen demonstrates that the antigen is able to confer high levels of protection against challenge infection with *T. solium*. Furthermore, the study indicate that the TSOL16 antigen could be a valuable adjunct to porcine vaccination with TSOL18 and may allow the further development of new vaccination strategies against *T. solium* cysticercosis (Gauci *et al*. 2012).

DNA vaccination is an exciting new strategy for the development of non-living sub-unit vaccines because of the generic nature of the production and purification processes required. This technique based on the use of recombinant DNA plasmids encoding specific antigens has been actively developed in the past few years against a variety of infectious agents including virus, bacteria and parasites (da Silva *et al*. 2014). Paramyosin, also known as B antigen, was identified as a candidate antigen for helminth vaccines and expressed specifically in the metacestode stage of *T. solium*. Protection rate in pigs with the DNA vaccine B-PV93 was reported to be up to 99.5%, substantially higher than that in the control. Immunization of pigs with 1000 µg of pcDNA3-B showed 92.6% protection when the pigs were challenged by *T. solium* eggs and proved to be useful in the prevention of cysticercosis (Guo *et al*. 2007).

The S3P vaccine is expressed recombinantly in M13 filamentous phage (S3Pvac-Phage) and provides high levels of protection against pig cysticercosis under experimental conditions. S3Pvac-Phage significantly reduced the prevalence of cysticercosis among the vaccinated pigs by 54.2% and, most significantly, reduced the intensity of infection with vesicular cysts by 87.1%. The efficacy of the S3Pvac-Phage vaccine reported similarly high to that obtained using the synthetic first version of the anti-cysticercosis vaccine (S3Pvac) (Morales *et al*. 2008).

Currently there is no registered drug for either treatment or prevention of cysticercosis, however studies have shown that porcine cysticercosis can be treated with oxfendazole (OXF), (Sikasunge *et al*. 2008, OIE Terrestrial Manual 2014), albendazole, prazinquatel (PZQ) and mabendazole (Kumar & Tadesse 2011). Cederberg *et al*. (2012) demonstrated that PZQ but not OXF or IVM had a significant effect on *T. solium* cysts *in vitro*. Even though no effect of IVM was observed *in vitro*, the effect *in vivo* cannot be rejected as it is used in many porcine cysticercosis endemic regions and have shown to be suitable for administration to free roaming pigs. More work is needed on efficacy and effectiveness of anthelmintics against *T. solium* in pigs in order to come forward with evidence-based control recommendations.

1.2 DIAGNOSIS OF TAENIOSIS IN HUMANS

Differentiation of *Taenia* species is important for epidemiological studies and for control of the diseases they cause. In humans, diagnosis of taeniosis is routinely performed by microscopic observation of the morphology of the tapeworm. The sensitivity of this method is low and also lacks specificity due to close similarities in both *T. saginata* and *T. solium* tapeworms (Chapman *et al*. 1995). Clear identification is done by examining the morphological features in the scolex and the proglottids of the two parasites. *Taenia saginata* shows more uterine branches in the proglottids than *T. solium*. It has 15 to 20 while *T. solium* has 7 to 13 uterine branches. The scolex of *T. saginata* lacks hooks (Figure 1.1A) while that of *T. solium* has hooks and double row of small hooks (Figure 1.1B) (Shore 2001).

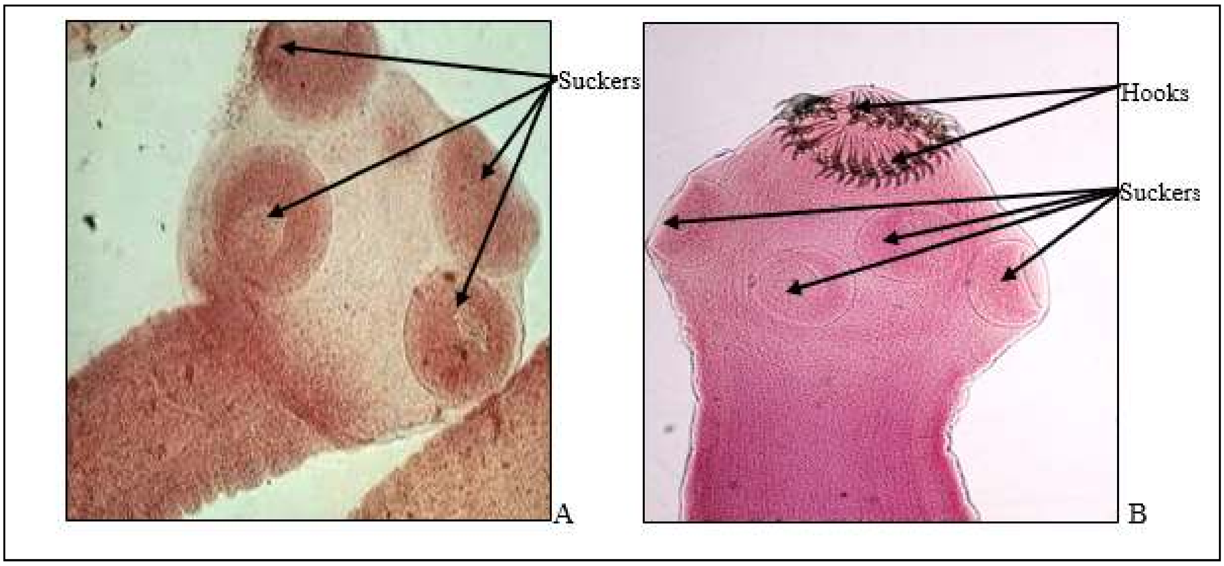


Figure 1.1: Scoleces of *Taenia saginata* (A) and *T. solium* (B). (Figures 1.1 A and B downloaded from http://www.vacunasyviajes.es/vacunasyviajes/Teniasis_Atlas.html and http://atlas.or.kr/atlas/alphabet_viewp?my_codeName=Taenia%20solium respectively).

1.3 DIAGNOSIS OF CYSTICERCOSIS IN CATTLE AND PIGS

Currently the diagnosis of bovine and porcine cysticercosis is through meat inspection, by examining the presence of cysts in different predilection sites such as the masseter, triceps muscles and heart muscles at the slaughterhouses (Figure 1.2). Meat inspection alone cannot eradicate the infection because of its low sensitivity which often leads to under-diagnosis, especially in the lightly infected carcasses. Furthermore, the skills and motivation of the meat inspector are important to the success or failure of an inspection. The heavily infected carcasses are rejected and totally condemned, while parts in the lightly infected carcasses are condemned and the rest of the carcass is frozen at a temperature lower than –10°C for >10 or 14 days, or lower than –7°C for 21 days to inactivate the parasites (OIE Terrestrial Manual 2014). Furthermore, the cysticercus can be killed by cooking all parts of the meat to a temperature above 56°C (Meiry *et al*. 2013). Even though the sensitivity of this method is high in detecting cysticercosis in the heavily infected carcasses, it is not reliable in detecting lightly infected positive carcasses are missed during meat inspection and passed on for human consumption (Minozzo *et al*. 2002; Kebede 2008). Consequently, meat inspection records tend to underestimate the disease prevalence. Furthermore, during meat inspection there is a possibility of mistaken identification of specific taeniid species involved due to cysts having died or degenerated or morphological similarities in lesions caused by taeniid larvae and other tissue parasites such as hydatid cysts and *Sarcocystis* spp. respectively (Gonzalez *et al*. 2006). The degenerated or calcified cysticerci can be confused with cysticerci of *T. hydatigena* (Sreedevi *et al*. 2012).

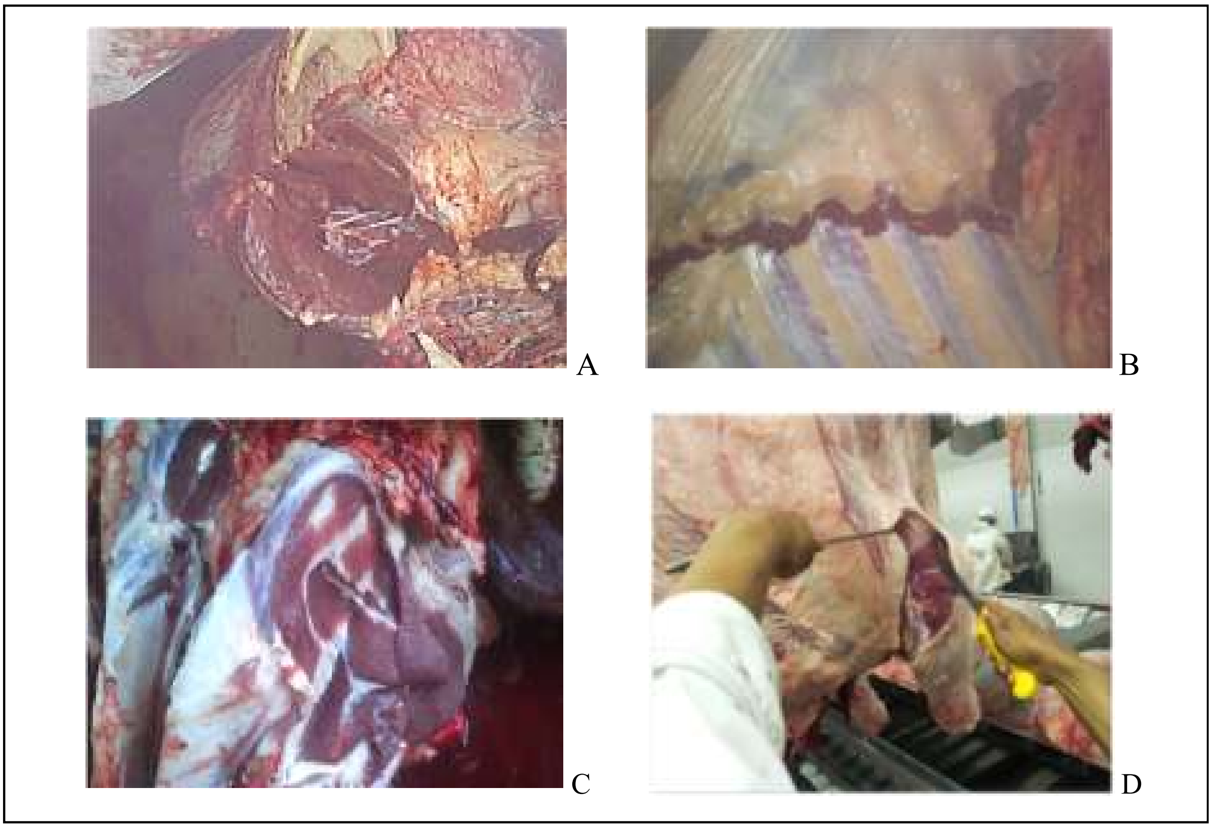


Figure 1.2: Meat inspection in various predilection sites: heart muscle (A), diaphragm (B), head muscle (C) and fore limb muscles (D). (Photos 1.2 A-C and D taken by E.S. Seipati and A.M. Tsotetsi respectively).

In Mbeya, Tanzania, prevalence of 6.6 % (16/243) of *T. hydatigena* was found in slaughtered pigs. The majority of cysts (80%) were found on the omentum and the rest on the liver (20 %), all on the visceral surface of the carcasses. In a total of 392 goats and 27 sheep examined by post-mortem, the prevalence of *T. hydatigena* was 45.7 and 51.9%, respectively (Braae *et al*. 2015).

The adult parasite has been reported in the small intestines of hosts including dogs, cats, mice and wild carnivores, like the wolf and the fox. The intermediate hosts are ruminants particularly sheep and wild ruminants. Infection with *T. hydatigena* is not pathogenic in dogs; however, its larvae migrate through the liver tissue of its definitive host and cause haemorrhagic and fibrotic tracts known as hepatitis cysticercosa (Kara & Doganay 2005). Cysticercus of *T. hydatigena* and a few migratory tracts of this larva are commonly seen in the offal of sheep and goats slaughtered. The most frequent unusual locations are in the lungs, the kidneys and the brain (Nourani *et al.* 2010).

Tongue inspection is also used to detect palpable cysts in pigs at the village level which may indicate porcine cysticercosis, however its low sensitivity reduces its utility as a diagnostic tool (Eshitera *et al.* 2012).

1.3.1 Morphology of *T. saginata* and *T. solium* cysticerci

The cysticerci are classified as alive or degenerated during meat inspection and become readily visible in infected carcasses from two weeks post infection (OIE Terrestrial Manual 2014). The cysticerci presenting white discolouration without distinct proto scolex are considered immature and the ones with proto scolex are matured (Minozzo *et al.* 2002). At week 4, the part of the cysticercus from which the scolex develops begins to evaginate, however there is still no evidence of suckers. At week 12 the larvae have the head retracted into a bladderlike structure and a developing neck (Figure 1.3A and B) (McIntosh & Miller 1960).

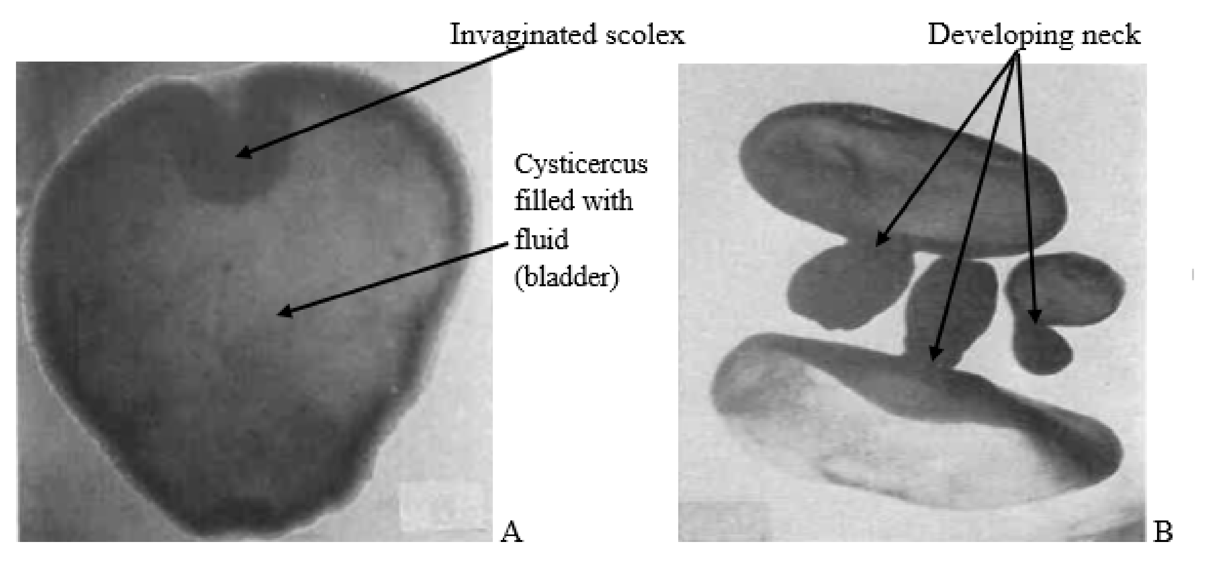


Figure 1.3: Taeniid cysticerci with invaginated scolex (A) and scolex with developed neck (B). (Figures taken from A. McIntosh & D. Miller 1960).

1.4 SEROLOGICAL ASSAYS

Immunodiagnostic tests have been developed for detection of specific antibodies or antigens/parasite products associated with current infection specific for *T. saginata* and *T.*

solium under natural and controlled conditions (Harrison *et al*. 1989; Smith *et al*. 1991; Brandt *et al*. 1992; Onyango-Abuje *et al*. 1996a, b; Dorny *et al*. 2000; 2002; Wanzala *et al*. 2002; Ferrer *et al*. 2003).They have gained acceptance as a tool for sero-epidemiological surveys (Onyango-Abuje *et al.,* 1996b; Dorny *et al.,* 2000, 2002).

Different antigens such as synthetic peptides, recombinant antigens, crude somatic extracts, excretory and secretory products have been used in the antibody detecting ELISA (Ferrer *et al*. 2003; Rhoads *et al*. 1991) while monoclonal antibodies that are produced against cyst fluid or excretory and secretory products of the cysts are used in antigen detecting ELISA (Harrison *et al*. 1989; Brandt *et al*. 1992).

Although these assays have been reported to be less sensitive in animals infected with fewer cysts, they have been shown to be three times more sensitive than meat inspection (Dorny *et al*. 2000; Geysen *et al*. 2007). The other advantage with the serological tests is that they are important tools for epidemiological studies since they can be used on live animals on large scale and the tests are inexpensive and easy to perform (Dorny *et al*. 2003). However, there are disadvantages related to serological tests that include measuring of the antigen exposure rather than actual infection, the interpretation of seropositive results in young pigs may be complicated by the transfer of the maternal antibodies from the sow to piglets (Gonzalez *et al*. 1999) and cross-reactions with other *Taenia* species (Dorny *et al*. 2003).

1.4.1 Antibody Enzyme-Linked Immunosorbent Assay (AbELISA)

Ferrer *et al*. (2003) evaluated six peptides derived from four potentially protective molecules cloned from a *T. saginata* oncospheres cDNA library as targets for the specific diagnosis of bovine cysticercosis. The six peptides consist of two peptides (HP6-2 and HP6-3) derived from the sequence of the 18 kDa surface/secreted oncospheral adhesion antigen identified by McAb-HP6, two peptides (Ts45W-1 and Ts45W-5) derived from the sequence of the *T. saginata* homologue of the *T. ovis* 45W protective gene family, one peptide (TS45S-10) derived from a *T. saginata* sequence with significant similarity to the *T. ovis* 45S protective antigen, and one peptide (TEG-1) derived from the sequence of the *T. saginata* homologue of *Echinococcus* spp. main surface protein. The study indicated that *T. saginata* infected cattle responded to all six peptides by 3-4 weeks post-infection and that the antibody levels remain high for at least 12 weeks post-infection. However, only three peptides (HP6-2, TEG-1 and Ts45S-10) showed necessary sensitivity and specificity that help in determining exposure to infection with *T. saginata*.

The five synthetic peptides, HP6-3, Ts45W-1, Ts45W-5, Ts45S-10 and TEG-1 derived from four, potentially protective, *T. saginata* oncosphere molecules evaluated previously in Ferrer *et al.* (2003) study were tested for the sero-diagnosis of *T. solium* cysticercosis/neurocysticercosis in three distinct Venezuelan endemic regions. The combined results of the three peptides showed the best balance between sensitivity of (85%) and specificity of (83.5%) (Ferrer *et al.* 2005).

Abuseir *et al.* (2007) used two similar peptides, HP6-2 and Ts45S-10 as antigens for the detection of antibodies against *T. saginata* cysticercosis in serum and meat juice samples using enzyme-linked immunosorbent assay (ELISA). Sensitivity and specificity of HP6-2 using serum were calculated as being 100 and 98% respectively, showing to be higher than the values for other antigens used. The peptide showed the highest sensitivity and specificity of 100 and 95% respectively in meat juices samples. The results of the study showed that the antibody ELISAs are sensitive and reliable methods for the determination of individual and herd prevalence rates of *T. saginata* cysticercosis.

Ferrer *et al.* (2007) evaluated the diagnostic utility of the *T. saginata* oncosphere adhesion protein (HP6-Tsag), expressed in baculovirus (HP6-Bac) and bacteria (HP6-GST [glutathione S-transferase]) for the detection of antibodies in sera from *T. saginata* infected cattle, *T. solium* infected pigs and serum and cerebrospinal fluid (CSF) samples from patients with *T. solium* neurocysticercosis (NCC). The two recombinant proteins were antigenic in all three systems, with the HP6-Bac ELISA slightly higher than that for the HP6-GST ELISA. Assays performance in cattle was similar whereas the sensitivity of the HP6-Bac and HP6-GST ELISAs was close for active human NCC (77.4 and 80.6% for serum and 76.9 and 73.1% for CSF samples, respectively). In inactive human NCC, however, the sensitivity of the HP6-Bac ELISA was almost twice that of the HP6-GST ELISA. Therefore, HP6-Tsag expressed in baculovirus (HP6-Bac) was regarded to be useful reagents for antibody detection in countries with endemic cysticercosis/NCC.

Ogunremi and Benjamin (2010) developed an enzyme-linked immunosorbent assay (ELISA) which used the excretory-secretory antigens of *T. saginata* to detect bovine anti-*T. saginata* immunoglobulin G1 antibodies. The test sensitivity was estimated to be 92.9% and specificity of 90.6%. All the animals that shown to harbour metacestodes at post-mortem tested positive in the ELISA. Since the assay can detect twice as many animals as the meat inspection procedure, it was suggested that the assay can be used in the feedlot with herds where an exposure to the parasite is expected. It can also be used in herds with history of *T. saginata* to determine which animals could be sent to slaughter.

Crude antigen of the whole *T. saginata* cysts used in the antibody detecting ELISA conducted by Kandil *et al*. (2012) outperformed the post-mortem examination of slaughtered cattle which showed 4% of infected cases with *T. saginata* cysticerci by revealing 29.3% seropositive samples from 75 slaughtered cattle. On the other hand, a similar antibody detecting ELISA using somatic crude *T. saginata* metacestode antigen (TsmAg) showed sensitivity of 77.7% and specificity of 87.9% with high cross-reactivity 68.75% against single *Fasciola hepatica*, and 56.5% against simultaneous *F. hepatica* and *Dicrocoelium dendriticum* infection in a study of Eichenberger *et al*. (2013).

Glycoproteins (GPs) purified and recombinant chimeric antigen (RecTs) of *T. solium* detected the antibody responses (IgG) in experimentally infected pigs after egg inoculation and in naturally infected pigs and harbouring 2.5 cysts/kg. Although pigs may be infected with other taeniid species such as *Taenia hydatigena*, pigs harbouring this parasite were negative in ELISA. Approximately, 76 and 78% of sera from pigs having nodules in the tongue (positive tongue inspection) were serologically positive by both ELISA and immunoblot, respectively. Furthermore, approximately 34 and 18% of sera from pigs having no nodules in the tongue (negative tongue inspection) were also seropositive by ELISA and immunoblot, respectively. ELISA using the two antigens was more sensitive than immunoblot and reliable for differentiation of pigs infected with cysticerci of *T. solium* from those either uninfected or infected with other taeniid species (Sato *et al*. 2003).

da Silva *et al*. (2012) reported the application of the MoAb anti -TS14 in the immunodiagnosis of porcine cysticercosis. The results obtained from the AgELISA performed by da Silva *et al*. (2012) demonstrated that the test is not appropriate for pigs with low infection when using monoclonal antibody against TS14 generated from the TS14 recombinant antigen as described in Greene *et al*. (1999) study. However, the test was successful in the naturally heavily infected pigs due to high infection rate compared to the experimentally infected pigs. The sensitivity of the test dropped from 92.3% to 12.8% when less than 50 cysticerci are present.

1.4.2 Antigen Enzyme-Linked Immunosorbent Assay (AgELISA)

Harrison *et al*. (1989) developed a double-sandwich ELISA based on the mouse monoclonal antibody (McAb) HP10 with a repetitive carbohydrate epitope on the lentil-lectin-adherent glycoprotein in the excretions and secretions of *T. saginata* cysticerci. This epitope is recognised and detected by the mouse monoclonal antibody (McAb) HP10 in serum of *T. saginata* infected cattle. The assay detected the antigen in serum from 4-5 weeks post-infection onwards and was associated with a current infection. The same assay also detected *T. solium* cysticercosis in humans.

Sciutto *et al*. (1998) confirmed that the HP10 AgELISA can also be used to detect *T. solium* cysticercosis. Both antigens and antibodies were detected early and at higher levels in heavily infected pigs than in lightly infected pigs. The presence of antigens bearing the HP10 epitope originally identified in *T. solium* and *T. saginata* was also verified in *T. crassiceps*.

Wanzala *et al*. (2002) identified 72% of the 25 naturally infected cattle as seropositive and 9/24 (37.5 %) of the artificially infected calves using the same assay.

Brandt *et al*. (1992) developed the MoAb-based antigen detection ELISA assays using MoAb antibodies 158C11 and 60H8. The two MoAb antibodies of the IgG1 isotype were produced against the secretion and excretion products of *T. saginata* cysticerci. The assay yielded a sensitivity of 92% and a specificity of 98.7% in sera of cattle harbouring more than 50 viable cysts. The assay detected only 12.8% of animals carrying less than 50 viable cysts. The sensitivity of both AgELISA assays indicated that they failed to detect animals with light infections. Similar reactions were obtained in animals harbouring only dead cysticerci and non-infected control animals. Cross-reactions were only observed with taeniid parasites. The test was able to detect circulating antigen also in sheep and pigs, respectively infected with *T. ovis* and *T. solium* and in the serum samples of confirmed cases of human *T. solium* cysticercosis (Brandt *et al*. 1992). Later (Dorny *et al*. (2000) reported suitable use of the (McAb) B158/B60 as an antigen capture assay when comparing and evaluating the diagnostic test characteristics of the available ELISAs.

Van Kerckhoven *et al* (1998) later improved the technique by developing a modified sandwich ELISA which used monoclonal antibodies (MAbs) of the IgG isotype, IgM- and IgG against the excretory-secretory (ES)-products of T. *saginata* metacestodes. Only a small percentage of animals carrying less than 50 cysts were detected both with the ELISA using IgG or IgM monoclonal antibodies. The specificity of the IgM- and IgG MAb-based ELISAs was 93.4% and 98.7% respectively. Dorny *et al*. (2000) performed the AgELISA as described by Van Kerckhoven *et al*. (1998) as a tool for a sero-epidemiological study of *T. saginata* cysticercosis in cattle slaughtered in the abattoirs. The sero-prevalence found in this study was more than 10 times higher than the annual prevalence (0.26%) reported. The study further indicated that the classical meat inspection techniques detect only a minor fraction of the carcasses infected with cysticerci.

1.5 MOLECULAR ASSAYS

Molecular diagnostic methods have been developed for the rapid, high sensitive and accurate detection of *Taenia* species (Harrison *et al*. 1990; Gonzalez *et al*. 2000, 2002; Yamasaki *et al*. 2004; Geysen *et al*. 2007; Chiesa *et al*. 2010).

There is no single set of conditions that is optimal for all PCRs, each PCR requires specific optimization for the primer pairs chosen or designed. It is important to optimize PCR that will be used for diagnostic or analytical procedures where optimal amplification is required (Grunewald 2003). Magnesium chloride as one of the components of PCR master-mix is critical to a successful of PCR amplification because it may affect DNA polymerase activity and fidelity, DNA strand denaturation temperatures of template and PCR product, primer annealing, PCR specificity, and the formation of primer-dimer. Excess Magnesium results in accumulation of nonspecific amplification products seen as multiple bands on an agarose gel, whereas insufficient magnesium results in reduced yield of the desired PCR product (Harris & Jones 1997; Williams 1989). It is important to optimize the Magnesium concentration used for PCR because DNA polymerases require free magnesium for their activity in addition to that bound by template DNA, primers, and dNTPs. Lack of PCR optimization results in problems, such as failure in producing the PCR product or low efficiency of amplification; the presence of nonspecific DNA bands or smeary background; the formation of primer-dimers; or mutations caused by errors in nucleotide incorporation. Furthermore, the quality of extracted DNA is very important for a successful PCR assay and spectrophotometry is used to measure the concentration and the purity of DNA samples by assessing the amount of ultraviolet irradiation that is absorbed by the bases of the DNA. The $OD_{260/280}$ ratio of 1.8-2.0 indicates that the absorption is due to nucleic acid (Desjardins and Conklin, 2010) while the $OD_{260/280}$ ratio greater than 2.0 indicates RNA contamination (Desjardins & Conklin 2010).

Amplification of the HDP1 and HDP2 DNA probes through polymerase chain reaction (PCR) allowed rapid and easy differential diagnosis of *T. saginata and T. solium*. (Gonzalez *et al*. 2000; 2010).

Analysis of the inter-species differences based on the sequence of the HDP2 DNA fragment which is specific for *T. saginata* and *T. solium,* and the sequence of the ribosomal DNA internal transcribed spacer 1 and spacer 2 (ITS-1 and ITS-2) resulted in unique PCR-RFLP pattern of isolates from different geographical areas (Gonzalez *et al*. 2002).

Sequence characterized amplified region (SCAR) markers were also used for differential diagnosis of *T. saginata* and *T. solium.* These SCAR markers were produced by Random amplified polymorphic DNA (RAPD) assays, used as genetic markers and regarded as potential tools to differentiate these parasites in epidemiological studies (Dias *et al*. 2007).

A number of molecular assays targeting the mitochondrial cytochrome c oxidase subunit I (*cox*1) gene were also developed. Yamasaki *et al*. (2004) developed a multiplex PCR that differentially diagnosed the causative agents of taeniosis and cysticercosis. The assay target cytochrome c oxidase subunit 1 gene with the forward primers specific for *T. saginata*; *T. asiatica*, *T. solium* African/American genotype and *T. solium* Asian genotype and a reverse primer common to all species. Coupling of the method with restriction fragment length polymorphism also appeared to be useful for differentiation of geographical isolates of *T. saginata and T. solium*. The method produced different genotypes unique for *T. saginata, T. asiatica* and *T. solium*. The assay was also considered being useful for molecular epidemiological survey of these cestode infections and control of cysticercosis (Yamasaki *et al*. 2004).

Chiesa *et al*. (2010) further developed biomolecular assay targeting the mitochondrial cytochrome c oxidase subunit I gene (COI) which proved to be suitable for samples containing both viable and degenerating *T. saginata* cysticerci producing clear diagnosis of *Taenia* infection. On the other hand, Sreedevi *et al*. (2012) developed assays that identified *T. solium* cysticerci with oligonucleotide primers specific to large subunit ribosomal RNA gene (TBR) and cytochrome c oxidase subunit 1 (*cox*1) gene which respectively yielded 286 and 984 bp products. Primers targeting large subunit ribosomal RNA gene were selected to identify and amplify degraded DNA in degenerated and calcified cysts/lesions. The detection limits of the PCR test with TBR primers and *cox*1 primers using the larval (metacestode) stage of *T. solium* DNA extracted by high salt method was 10 pg and 1 ng respectively.

Although the conventional PCR is a powerful tool in the detection and identification of parasites, it has its limitations in quantitative analysis. Real-time PCR technique offers the ability to quantify the initial target DNA in one reaction. The assay detects and measures PCR products generated during each cycle of the reaction (Navarro *et al*. 2015). The labelling of the oligonucleotide probes with fluorescent dyes has made the monitoring of the accumulation of the PCR amplicons possible. The fluorescent signal produced during the increase of the specific PCR products matches up with the amount of the amplification products produced during each cycle. Real-time PCR has been regarded as a powerful diagnostic tool for rapid, sensitive and quantitative detection of different pathogens (Al-Soud & Radstrom 1998).

Cuttel *et al*. (2013) developed real-time PCR for reliable and rapid laboratory confirmation method to detect DNA of *T. saginata* suspect cysts encountered at meat inspection and compared its use with the traditional method of identification. The assay detected *T. saginata* DNA using the cytochrome c oxidase subunit 1 gene and showed specificity against parasites causing lesions morphologically similar to those of *T. saginata*. Specificity of this developed real-time PCR showed no cross-reactivity in genomic DNAs from other parasite species such as *Echinococcus granulosus*, *Taenia hydatigena*, *Spirometra erinacei*, *Toxocara vitulorum*, *Toxocara canis*, *Sarcocystis cruzi*, *Toxoplasma gondii*, *Neospora caninum* and *Actinobacillus lignieresii* that may occur in beef muscle.

The assay was sufficiently sensitive to detect target DNA in all viable and caseated positive control cysts. The limit of detection using the DNA standard was 220 copies of target gene or 1 fg of *T. saginata* DNA corresponding to a Ct value of 35.09**.** However, cyst degeneration resulted in decreased PCR sensitivity in the suspect cysts. Ct scores in some calcified and highly degenerated samples were around or greater than the limit of detection supporting a loss of PCR sensitivity with sample degeneration. Real-time PCR assay outperformed histological examination in both sensitivity and specificity as histology failed to positively identify any *T. saginata* cysticerci. A total of (53%) 10/19 *T. saginata* positives were detected whereas none of the suspect cysts could be identified by histology. The use of the cysticerci represented a spectrum of viability and the assay detected 100% of viable and caseated cysts, but failed to detect 1/16 calcified and 2/3 highly degenerated samples. Even though the assay was less sensitive compared to conventional PCR, it offered advantages of faster turnaround times and reduced contamination risk (Cuttel *et al*. 2013).

Praet *et al.* (2013) developed a qPCR targeting the internal transcribe spacer 1 (ITS1) genes of each of *T. solium* and *T. saginata* and detects these *Taenia* species in faeces. With these ITS 1 qPCR assays the lowest amount of *T. solium* and *T. saginata* DNA amplified was estimated at 1 and 2.5 fg respectively. Performance of each assay was not influenced by the presence of

DNA from other cestodes. Amplification of *T. solium* and *T. saginata* were both detected at the Ct values between 21.9 and 37.7 with a median Ct value of 27.8 cycles. Specificity and sensitivity of the ITS1 qPCR assays were reported as 99.0% and 82.7% respectively. The detection of the tapeworm carriers with the assay was seen as a key factor in controlling the parasite in endemic areas (Praet *et al*. 2013).

The limitations of the current meat inspection procedure pose significant challenges for regulators and diagnostic tasked with preventing zoonotic transmission of the parasite. The diagnosis of infected animals is currently carried out in the slaughterhouse by meat inspection. The method is effective in detecting heavily infected carcasses, but not reliable at detecting lightly infected carcasses. During meat inspection, there is also a possibility for mistaken identifications due to cysts having died and degenerated or due to macroscopic morphological similarities in lesions caused by taeniid larvae and other tissue parasites, such as Sarcocystis species.

CHAPTER 2: AIMS AND OBJECTIVES OF THE STUDY

2.1 STATEMENT OF THE PROBLEM

Bovine and porcine cysticercosis cause huge economic losses in beef and pork industry due to condemnation of infected beef and pork carcasses and low quantity of beef and pork supply (Qadeer 2008; Megersa *et al*. 2010; Sciutto *et al*. 1998).

Currently diagnosis of bovine and porcine cysticercosis is mainly by meat inspection which although being useful in detecting cysticercosis in heavily infected carcasses, lightly infected carcasses may be easily missed and passed on for human consumption. As a result, the use of meat inspection records tends to underestimate the disease prevalence. On the other hand, during meat inspection, there is also a possibility for mistaken identifications due to cysts having died and degenerated or due to macroscopic morphological similarities in lesions caused by taeniid larvae and other tissue such as *Sarcocystis* species (Gonzalez *et al*. 2006).

Serological tests have been developed for the detection of specific antibodies or antigens specific to *T. saginata* and *T. solium* and the detection of the parasite products associated with current infection (Onyango-Abuje *et al.,* 1996b; Dorny *et al.,* 2000, 2002). Although these assays have been reported to be less sensitive in animals infected with fewer cysts, they have shown to be three to 10 times more sensitive than meat inspection and have been successfully used in the epidemiological surveys (Dorny *et al*. 2000; Geysen *et al*. 2007). However, these assays are not species specific which makes it difficult to obtain clear diagnosis of taeniid infections. Furthermore, taeniid species have different impact on human beings, with *T. solium* being the most important in causing fatal neurocysticercosis (Gonzalez *et al*. 2006). It is therefore very important to accurately and specifically diagnose the species involved. Accurate diagnosis of infectious diseases is a very important part leading to proper treatment, control and even elimination of the disease. The limitations of the current diagnostic tools create significant challenges for regulators and technicians in preventing zoonotic transmission of the parasites from human to animals and from animals to humans (Gonzalez *et al*. 2006). The study therefore aimed at employing the currently available monoclonal antibody HP10 antigen detecting ELISA and conventional PCRs to respectively detect and confirm *Taenia* cysticercosis in cattle brought for slaughter at abattoirs in Free State Province. *T. saginata* and *T. solium* real-time PCR assays were also developed.

2.2 AIMS AND OBJECTIVES

The study was aimed at developing and adapting specific and sensitive assays for detection of bovine and porcine cysticercosis.

2.2.1 General and Specific objectives

1. **To optimise and to use conventional PCR assay to confirm *Taenia saginata* and *T. solium* cysticerci identified during meat inspection and to detect *T saginata* and *T. solium* in live cattle and pigs**

Specific objectives

i. To optimise HotStar *Taq* and My *Taq* PCR assay conditions with previously designed and published primers by (Gonzalez *et al*. 2000) for detection of HDP2 gene in cyst samples for differential diagnosis of *T. saginata* and *T. solium*.

ii. To optimise HotStar *Taq* and My *Taq* PCR assay conditions with previously designed and published primers by (Nkouawa *et al*. 2009) for detection of *cox*1 gene in cyst samples for differential diagnosis of *T. saginata* and *T. solium*.

iii. To use the successfully optimised PCR assay to confirm identifications of *T. solium* and *T. saginata* cysticerci made during meat inspection.

iv. To use the successfully optimised PCR assay to detect *T. saginata* and *T. solium* infections in blood samples collected from cattle and pigs at abattoirs.

2. **To develop real-time PCR assay for detection of *T. saginata* and *T. solium* infections in cattle and pigs respectively.**

Specific objectives

i. To design primers and probes specific to *T. saginata* and *T. solium cox*1 gene.

ii. To determine the detection limit of the *T. saginata* and *T. solium* real-time *Taq*Man PCR assays.

iii. To determine the specificity of the *T. saginata* and *T. solium* real-time *Taq*Man PCR assays.

iv. To validate the use of newly developed *T. saginata* and *T. solium* real-time *Taq*Man PCR assays in confirming cysts identification.

v. To validate the use of newly developed *T. saginata* and *T. solium* real-time *Taq*Man PCR assays for the detection of *T. saginata* and *T. solium* infections in bovine and porcine blood samples respectively.

3. To determine the prevalence of bovine cysticercosis using the McAb (HP10) antigen detecting ELISA for detection of *T. saginata* infections in cattle.

Specific objectives

i. To use the (McAb) HP10 AgELISA to determine prevalence of bovine cysticercosis in cattle brought for slaughter in Free State abattoirs.

ii. To compare prevalence determined by the McAb (HP10) AgELISA to meat inspection results.

CHAPTER 3: MATERIALS AND METHODS

3.1 Optimisation and the use of conventional PCR assay for confirmation of *Taenia saginata* and *T. solium* cysticerci identifications made during meat inspection and detection of *Taenia saginata* and *T. solium* in live cattle and pigs

3.1.1 Optimisation of conventional PCR assay

3.1.1.1 Sources of DNA

For optimisation purposes, DNA previously extracted by phenol chloroform method from a *T. saginata* tapeworm was used for *T. saginata* assay. DNA extracted from *T. solium* cysticercus and porcine blood samples collected from a Free State abattoir were used for optimisation of the *T. solium* assay.

3.1.1.2 DNA extraction

Three DNA extraction methods were used; Phenol chloroform method, High Pure PCR Template Preparation (Roche) and QIAamp DNA Mini (Qiagen) kits.

a. Phenol chloroform extraction method (Sambrook *et al.* 1989)

Blood sample at a volume of 50 µl or small pieces of *T. saginata* proglottid were lysed with 500 µl of lysis buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate followed by the addition of 10 µl of the commercial Proteinase K. Samples were lysed overnight in the heating block at 55°C. On the second day 550 µl of phenol chloroform was added to the reaction tubes followed by mixing and centrifuging at 15 000 rpm for 5 minutes. The upper aqueous phase was added to 500 µl of Chloroform, mixed and centrifuged at 15 000 rpm for 5 minutes. The upper aqueous phase was added to 50 µl of Sodium acetate (3 M NaOAc) and 500 µl of Isopropanol in the new tube and the mixture was centrifuged for 30 minutes at the speed of 15 000 rpm. The pellet was washed with 70% Ethanol twice and mixed and centrifuged at 15 000 rpm for 5 minutes after each wash. The tube containing the pellet was left open for 2 minutes at room temperature and dissolved in 150 µl of TE buffer and incubated at 37°C for 1 hour before it could be stored at –20°C until future use in PCR amplification.

b. High pure PCR template preparation (Roche Diagnostics, Mannheim, Germany)

Small pieces of *T. saginata* proglottid were digested in the Eppendorf tube containing 200 µl tissue lysis buffer of the DNA extraction kit. Then 40 µl of Proteinase K was added followed by the immediate mix. Binding buffer (200 µl) was added, and the mixtures were mixed and incubated at 70°C for 10 minutes. Isopropanol (100 µl) was added and the 1000 µl pipette was used to withdraw the insoluble tissue particles. The liquid samples were pipetted in the collection tube assemble and spun down at 8 000 x g for 1 minute. The columns were then washed twice with 500 µl of wash buffer (Roche) and centrifuged after each wash step. The extracted DNA was eluted in 200 µl of pre-warmed elution buffer into new sterile 1.5 ml Eppendorf tubes and stored at –20°C until PCR amplification could be performed.

c. QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany)

Genomic DNA was isolated using the QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany) according to the manufacturer's protocol for DNA isolation from cysts. Small pieces of *T. saginata* and *T. solium* cyst samples were lysed with 180 µl of ATL (tissue lysis buffer) and 20 µl of Proteinase K was added and tubes were vortexed to mix DNA pellets in the solution and incubated at 56°C until the cells are completely lysed. For blood samples, 20 µl of Proteinase K was added to the bottom of a 1.5 ml microcentrifuge tube then 200 µl whole blood was added to the microcentrifuge. Then 200 µl of Buffer AL was added to both blood and cyst samples and mixed by pulse-vortexing for 15 seconds. Then the tubes with cyst samples were incubated at 70°C and those with blood samples were incubated at 56°C and all tubes were incubated for 10 minutes. Then 200 µl of ethanol (96-100%) was added and mixed thoroughly. The mixture was then pipetted to the QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute and the tube containing the filtrate was discarded. QIAamp Mini spin columns were placed in new 2 ml collection tubes, 500 µl Buffer AW1 (wash buffer 1) was added and centrifuged for 1 minute at 8000 rpm. The tube containing the filtrate was discarded, QIAamp Mini spin columns were placed in another new 2 ml collection tubes, 500 µl Buffer AW2 (wash buffer 2) and centrifuged for 1 minute at 14,000 rpm for 3 minutes and tubes containing the filtrate were discarded. QIAamp Mini spin columns were placed in new 2 ml collection tubes and centrifuged at full speed for 1 minute and tubes containing the filtrate were discarded. Spin column tube was kept in a new 1.5 ml or 2 ml micro-centrifuge tube and 200 µl AE (elution buffer) buffer added directly on the QIAamp membrane. These tubes were incubated at room temperature for 1 minute and centrifuged at 6000 x g (8000 rpm) for 1 minute to elute DNA. Extracted DNA was preserved at –20°C.

3.1.1.3 Quantification and quality of extracted DNA

The concentration and quality of the DNA extracts were measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific) depicted in Figure 3.1. The optical densities (OD) were measured at wavelengths of 260 and 280 nm to record the purity and concentration of DNA in ng/ µl.

Figure 3.1: NanoDrop 1000 spectrophotometer (Thermo Scientific). (Photo taken by E.S. Seipati 2014).

3.1.1.4 Polymerase Chain Reaction (PCR) assays

Two sets of primers were used and tested according to Gonzalez *et al*. (2000) study targeting the HDP2 gene and Nkouawa *et al*. (2009) targeting the mitochondrial *cox*1 gene.

A. HDP2 gene (Gonzalez *et al*. 2000)

a. Primers for the HDP2 gene

The PTs7S35F1 and PTs7S35R1 primers were used to amplify the product length of 600 bp in the HDP2 gene while the product length of 170 bp was amplified with PTs7S35F2 and PTs7S35R1 primers. Sequences of the primers are depicted in Table 3.1.

Table 3.1: Primer sequences for amplification of the 600 bp and 170 bp HDP2 genes.

Species	Primer set	Sequence (5' to 3')	Product length
T. saginata	PTs7S5F1	5'-CAGTGGCATAGCAGAGGAGGAA-3'	600 bp
	PTs7S35R1	5'-GGACGAAGAATGGAGTTGAAGGT-3'	
T. saginata	PTs7S35F2	5'-CTTCTCAATTCTAGTCGCTGTGGT-3'	170 bp
and *T. solium*	PTs7S35R1	5'-GGACGAAGAATGGAGTTGAAGGT-3'	

b. PCR conditions for detection of the HDP2 gene

All PCR reactions were carried out in 0.2 ml PCR tubes (Whitehead Scientific) in a Labnet MultiGene thermal-cycler (Figure 3.2) using the thermal cycling profile depicted in Table 3.2.

Figure 3.2: Labnet MultiGene thermal-cycler. (Photo taken by E.S. Seipati 2014).

Thermal cycling conditions in Table 3.2 were used exactly as described by Gonzalez *et al.* (2000) with number of cycles kept at 35 except for annealing temperature of primers. Different annealing temperatures ranging between 53 and 58°C were tested in HotStar *Taq* and My *Taq* PCR assays to determine the best annealing temperature for amplification of the 600 bp HDP2 gene.

Table 3.2: Cycling conditions for amplification of HDP2 gene using HotStar *Taq* and My *Taq* PCR assays.

Steps	Cycles	Time	Temperature
Initial denaturation	1 cycle	5 minutes	94°C
Amplification	35 cycles		
Denaturation		10 seconds	94°C
Annealing		30 seconds	53-58°C
Extension		30 seconds	72°C
Final extension		10 minutes	72°C
Cooling	1 cycle	10 seconds	4°C

c. Concentrations of the HotStar *Taq* PCR assay mix

Different $MgCl_2$ concentrations ranging between 1.5 mM to 5 mM were used for the detection of the target genes in the *T. saginata* positive control sample. Separate master mixes were prepared, one with PTs7S35F1 and PTs7S35R1 primers for amplification of the 600 bp HDP2 gene and the other with PTs7S35F2 and PTs7S35R1 primers for detection of the 170 bp HDP2 gene. Since PCR of the present study was a single step reaction, the concentrations of the primers were adjusted to 10 µM instead of 0.5 µM used in the multiplex PCR reaction described in the Gonzalez *et al*. (2000) study. The target genes were detected from the 2 µl template. The prepared HotStar *Taq* PCR master-mixes were performed in a total volume of 25 µl of reaction mixture containing 10x PCR buffer (Qiagen) and $MgCl_2$ (Qiagen) at different concentrations ranging from 1.5 mM to 5 mM, 200 mM of dNTPs mix (Bioline), 10 µM of each forward and reverse primer, 2.5 U of *Taq* polymerase (Qiagen) and 2 µl of the template with their volumes depicted in Table 3.3.

Table 3.3: Reaction setup for HotStar *Taq* PCR assay mix.

Reaction mix Components	**Volume/reaction (µl)**
Buffer 10x	2.5
Template	2
Taq	0.125
dNTPs (10 µM)	0.5
Reverse Primer	1
Forward Primer	1
MgCl 25 mM (1 mM)	3
Distilled water	14.875
Total reaction volume	**25**

d. Concentrations of My *Taq* PCR assay mix

My *Taq* PCR prepared master-mixes contained 2 µl of DNA template, forward and reverse primer each, 1x PCR buffer (Bioline) with optimised concentrations of the dNTPs and $MgCl_2$ and with their volumes indicated in Table 3.4.

Table 3.4: Reaction setup of My *Taq* PCR mix.

Reaction mix Components	**Volume/reaction (µl)**
Buffer 10x	12.5
Template	2
Reverse Primer	1
Forward Primer	1
Distilled water	8.5
Total reaction volume	**25**

B. Mitochondrial *cox*1 genes (Nkouawa *et al*. 2009)

a. Primers for the *cox*1 gene

The *Tsag-cox*1 F3 and *Tsag-cox*1 F3 primers were used to amplify the *cox*1 (238 bp) gene in *T. saginata* tapeworm and *Tsol-cox*1 F3 and *Tsol-cox*1 F3 primers were used to amplify the *cox*1 (211 bp) gene (Table 3.5) in *T. solium* cysticercus.

Table 3.5: Primer sequences for amplification of *T. saginata* and *T. solium cox*1 genes.

Species	**Primer set**	**Primer type**	**Sequence (5' to 3')**	**Product length**
T. saginata	*Tsag-cox*1	F3	TCGGCAAATATTTAATTCCT	238 bp
		B3	AAATTCTAGACGCACCCG	
T. solium	*Tsol-cox*1	F3	CCTATTTTAATTGGAGGTTTTGG	211 bp
		B3	CTACCCCACTTCCTCTTGA	

b. PCR conditions for detection of the *cox*1 gene

HotStar *Taq* and My *Taq* PCR assays were also used for amplification of the 238 bp *T. saginata* and 211 bp *T. solium cox*1 genes following the thermal cycling profile indicated in Table 3.6 with an annealing temperature of 58°C as described in Nkouawa *et al*. (2009).

Table 3.6: Cycling conditions for amplification of *T. saginata* and *T. solium cox*1 gene.

Steps	**Cycles**	**Time**	**Temperature**
Initial denaturation	1 cycle	5 minutes	94°C
Amplification	35 cycles		
Denaturation		10 seconds	94°C
Annealing		30 seconds	58°C
Extension		30 seconds	72°C
Final extension		10 minutes	72°C
Cooling	1 cycle	10 seconds	4°C

c. Concentrations of My *Taq* PCR assay mix

The 1x buffer contained the optimised concentrations of $MgCl_2$, dNTPs and *Taq* polymerase. The 10 µM concentration was used for each primer which was different from the 0.2 µM used in Nkouawa *et al*. (2009) study. My *Taq* PCR assay mixes for detection of the *cox*1 genes were prepared as indicated previously in Table 3.4. However DNA template of 0.5 µl was used instead of 2 µl which was used in amplification of the HDP2 gene. My *Taq* PCR assay was also used for optimisation of the target gene in DNA extracted from one of the porcine blood samples collected from the Free State abattoirs. Different volumes between 0.5 and 5 µl of DNA were used for optimisation of detection of the target *cox*1 gene.

d. Concentrations of the HotStar *Taq* PCR assay mix

The reagents for HotStar *Taq* PCR assay master-mixes were prepared as previously indicated in Table 3.3 and $MgCl_2$ concentration was at 3 mM. The *cox*1 gene was amplified from 0.5 µl of the DNA template in HotStar *Taq* PCR assay.

3.1.1.5 Determination of the detection limit (sensitivity) of the PCR assay

Tenfold serial dilution was done on the *T. saginata* and *T. solium* positive control samples with concentrations of 130 ng/µl and 34 ng/µl respectively. *Taenia saginata* DNA concentration 100 ng/µl was serially diluted in 8 tubes and the DNA concentration was decreased from 100 ng/µl to 1 fg/µl using distilled water. The concentration of *T. solium* was also serially diluted in 7 tubes from 10ng/µl to 1 fg/µl. The different concentrations were tested in PCR assays as previously described in order to determine the lowest DNA concentration of the *T. saginata* and *T. solium* target *cox*1 genes that can be detected in their respective assays.

3.1.1.6 PCR analysis

The amplified PCR products were analysed by gel electrophoresis. Two microliters of each PCR product was mixed with 8 µl of 6x loading dye (Promega) and loaded on the 2% agarose gel containing ethidium bromide. The DNA was electrophoresed through the Agarose gel at 100Volts for 30 minutes using a BioRad electrophoresis (Figure 3.3A). The products were visualised under UV light and recorded by a BioRad gel documentation system (Figure 3.3B). The sizes of the DNA bands were verified by running the 100 bp DNA ladder (Promega) in parallel with amplicons along with PCR products.

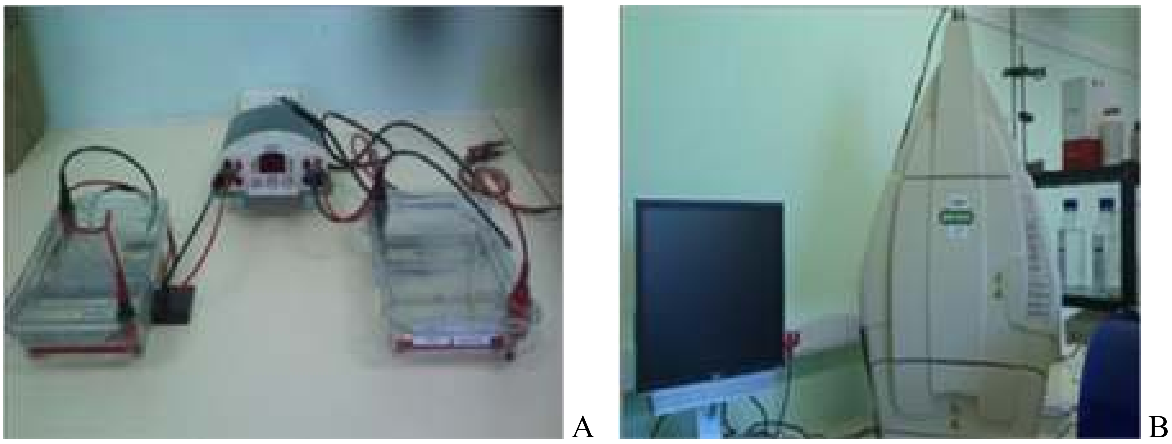


Figure 3.3: Gel tank and power supply (A) and BioRad gel documentation system (B). (Photos taken by E.S. Seipati 2014).

3.1.2 Validation of PCR assays using field samples

3.1.2.1 Study area

Blood and cyst samples were collected in cattle and pigs at five districts of Free State Province which are Thabo Mofutsanyana, Xhariep, Motheo, Fezile Dabi and Lejweleputswa (Figure 3.4). Abattoirs were identified through the assistance of the Free State provincial veterinary services. Geographical coordinates of each determined site were recorded and later used for mapping of specific sites where samples were collected.

Figure 3.4: Map showing five districts of Free State Province. (Figure downloaded from http://aoin.eu/wiki/index.php?title=Orange_Free_State).

3.1.2.2 Collection of cyst samples

Meat inspection was performed by the abattoir meat inspectors who examined the tongue, diaphragm, heart, masseter and triceps muscles for the presence of *Taenia* cysticerci. Each cyst sample was dissected from the muscles of the carcasses, stored separately in 100% Ethanol in sterile specimen collection containers and transported to ARC-OVI lab where they were stored at –20°C until further use.

3.1.2.3 Collection of blood samples

Blood sample were collected in the 6 ml lavender BD VACUTAINER® EDTA (anticoagulant) tubes and 5ml venous blood vacutainer after slaughtering of cattle and pigs (Figure 3.5) for molecular and serological analysis respectively. The supernatant serum from the 5ml venous blood vacutainer was transferred into 1.40 ml U-bottom tube. All blood samples were stored in a cooler-box and transported to ARC-OVI lab where they were stored at –20° C until further use.

3.1.2.4 DNA extraction from blood and cyst samples

Blood samples were extracted with phenol chloroform method and cyst samples with Qiagen kit according to conditions described in Appendix 1. PCR targeting mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene of *T. saginata* and *T. solium* was performed using the LabNet thermocycler under the conditions described in Table 3.6 and also in Appendix 2. The *Tsag-cox*1 (F3+B3) and *Tsol-cox*1 (F3+B3) primers previously designed by (Nkouawa *et al.* 2009) were used for amplification of the *cox*1 gene. All the amplified PCR products obtained from the amplification reactions were confirmed and analysed on 2% ethidium bromide stained agarose gel under U.V trans-illuminator of gel documentation system (Bio-Rad Gel Doc) and photographed as described in Appendix 3. Blood samples were pooled per abattoir prior to being tested of individually.

3.2 Development and validation of *Taq*Man real-time PCR assay for detection of *T. saginata* and *T. solium* infections in cattle and pigs respectively

3.2.1 Sources of control DNA used for assay development

The genomic DNAs extracted from a proglottid of *T. saginata* tapeworm and *T. solium* cysticercus were used in development of *T. saginata* and *T. solium* real-time *Taq*Man PCR respectively. The DNAs of *T. hydatigena* metacestodes, pig and cattle muscles were used as the negative controls. Nuclease free water was used as a no DNA negative control.

Figure 3.5: Collection of blood samples from animals brought to slaughter: Animals brought to slaughter (A), collection of blood from the slaughtered animals' throat (B), transfer of the blood sample from the sterile specimen bottle into VACUTAINER® EDTA tube (C) and blood in the VACUTAINER® EDTA tube (purple top) and 5ml venous blood vacutainer (yellow top). (Photos taken by A.M.Tsotetsi 2014).

3.2.2 DNA extraction

The genomic DNA from proglottid of *T. saginata* tapeworm was extracted with Phenol chloroform method (Sambrook *et al*. 1989) and DNA from *T. solium* cysticercus was extracted with QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany according to the manufacturer's instructions.

3.2.3 DNA quality and concentration

DNA quality and concentration were measured as described in 3.1.1.3

3.2.4 Primer design

The complete *Taenia saginata* and *T. solium* mitochondrial DNA sequences were downloaded from the NCBI GenBank with accession numbers AB821273.1 and AB781361.1. The sequences were sent to the Roche Company to design species specific primers and *Taq*Man fluorescence labelled probe for real-time PCR assay for each parasite.

The sequences of the primers and probe are listed in Tables 3.7 and 3.8. The *T. saginata* and *T. solium* specific probes were labelled with 6-carboxyfluorescein (FAM) at the 5' end and with N, N, N', N'-tetramethyl-6-carboxyrhodamine (BBQ) fluorescent quencher at the 3' end. The primers and probes were purchased from TIB MOLBIOL, Berlin, Germany.

Table 3.7: The primers and probe sequences of *T. saginata cox*1 gene.

Primer	Sequence (5' to 3')	Position
Saginata F	5'-TTCggCAAATATTTAATTCCTTTg-3'	241-264
Saginata R	5'-AAATTCTAgACgCACCCg-3'	478-461
Saginata TM	6FAM-AAAAgTCCACCCTATACCAgCACC-BBQ	388-364

Table 3.8: The primers and probe sequences of *T. solium cox*1 gene.

Primer	Sequence (5' to 3')	Position
Solium F	5'-CCTATTTTAATTggAggTTTTgg-3'	223-245
Solium R	5'-AACATTAAAAAATCTACCCCACTTCC-3'	446-421
Solium TM	6FAM-TgCATggTTGTTAgTTCCATCAATTgTTT-BBQ	312-341

3.2.5 Optimisation of *Taq*Man real-time PCR assay

3.2.5.1 Determination of PCR conditions for optimal annealing temperature of primers and probes

*Taq*Man real-time PCR reactions were performed on a LightCycler® 96 system (Roche Diagnostics, Mannheim, Germany). Amplification and detection were performed in three step amplifications under the thermal-cycling conditions stated in Table 3.9a and b. DNA extracted from *T. solium* cysticercus was used to determine an optimal annealing temperature of primers and probes for the assay at different temperatures between 55ºC and 58ºC.

Table 3.9a: Three-step thermal-cycling conditions of *Taenia saginata* and *T. solium* real-time PCR assays.

Program Name	Cycles	Analysis Mode
Pre-incubation	1	None
3 step Amplification	45	Quantification
Cooling	1	None

Table 3.9b: Three-step thermal-cycling conditions of *Taenia saginata* and *T. solium* real-time PCR assays.

Program	Temp °C	Hold	Ramp/rate °C	Acquisition Mode
Pre incubation	95ºC	10 min	4.4	None
Amplification	95ºC	10 sec	4.4	None
	55ºC - 58ºC	30 sec	2.2	None
	72ºC	30 sec	4.4	Single
Cooling	40ºC	10 sec	2.2	None

3.2.5.2 Preparation of the PCR master mix

Master-mixes for PCR reactions were prepared according to manufacturer's instructions as shown on Table 3.10 with a final volume of 20 µl and each experiment included reaction mixtures without DNA template as negative control.

Table 3.10: Reaction setup of *Taq*Man real-time PCR mix.

Reaction mix Components	Volume/reaction (µl)
2x LightCycler® 480 Probe Master reaction mix	10
Forward primer	2
Reverse primer	2
*Taq*Man probe	0.8
LightCycler® 480 Probe Master water PCR-grade	0.2
Template DNA	5
Total reaction volume	**20**

3.2.5.3 Determination of the detection limit of the assays

Taenia saginata and *T. solium* positive control samples were ten-fold serially diluted with distilled water from their respective initial concentrations of 130 ng/µl and 34 ng/µl as illustrated in Figure 3.6 below.

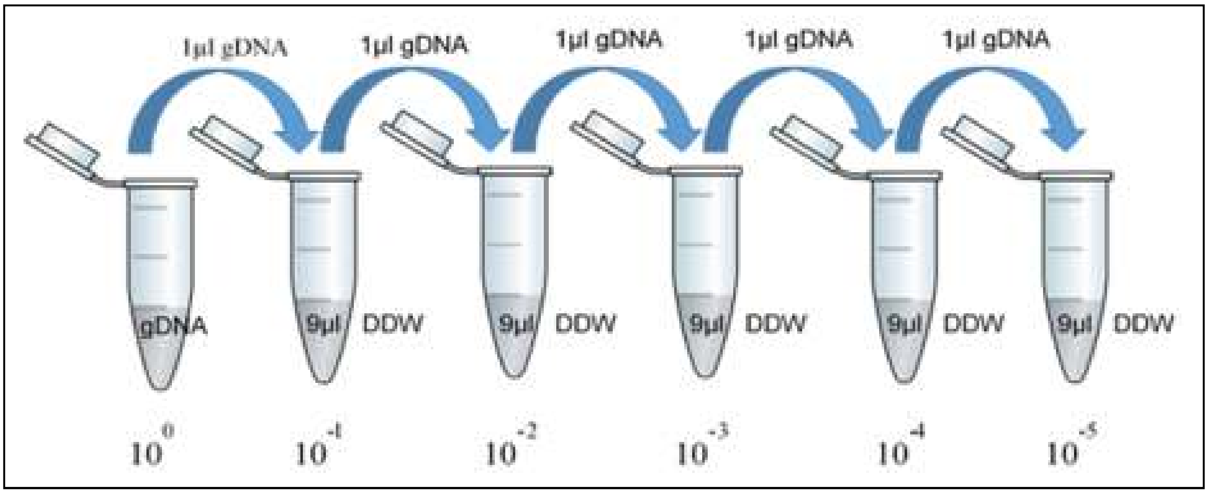


Figure 3.6: Stepwise dilution of a positive control of the *T. saginata* and *T. solium* DNA.

Reactions were conducted in triplicate and the standard curve was generated by plotting the Ct values against log DNA concentration. Linear regression was calculated by the LightCycler® 96 system Data Analysis software and reactions were performed under conditions explained previously in Tables 3.9 and 3.10.

3.2.5.4 Determination of the assays' specificity

The specificity of the *T. saginata* real-time PCR assay was tested against DNAs of related *Taenia* species; *T. solium* and *T. hydatigena* as well as pig and cattle muscles with reactions conducted in triplicate. The *T. solium* real-time PCR specificity was also tested against pig and cattle muscles and its related *Taenia* species which are *T. saginata* and *T. hydatigena* using the PCR conditions explained above.

3.2.5.5 PCR product analysis

LightCycler® 96 system (Roche Diagnostics, Mannheim, Germany) was used for analysing the results. The increase in fluorescent signal was monitored by the sequence detector as the reaction progressed. Positive samples were defined as those that demonstrated the fluorescent signal above the threshold line and positive reactions were given a cycle threshold Ct value equal to the cycle number where the fluorescence crossed the threshold line.

3.2.6 Validation of the PCR assays using field samples

3.2.6.1 Sources of field samples

DNA was extracted from both blood and cyst samples collected from cattle and pigs brought to slaughter at various abattoirs in the Free State Province (see Figure 3.5). This field-derived DNA was used in validation of the newly developed *T. saginata* and *T. solium* real-time PCR assays. Genomic DNA was extracted using phenol chloroform method (blood) and QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany (cysts) as described in 3.1.1.2. The extracted DNA was then analysed according to conditions of the newly developed assays described in Appendix 4.

3.3 Detection of *Taenia* infections in cattle using MoAb (HP10) antigen detecting ELISA

3.3.1 Study area

Blood samples were collected from areas/sites mentioned in section 3.1.2.1.

3.3.2 Meat inspection

Meat inspection was performed by the abattoir meat inspectors on the carcasses of the cattle that were brought for slaughter. The tongue, diaphragm, heart, masseter muscles of the head and triceps of the foreleg muscles were examined for the presence of the cysts as illustrated in Chapter 1. Records of meat inspection were kept by the abattoirs until further use.

3.3.3 Collection of blood samples

Blood samples were collected from cattle between March and August 2014. The samples were collected in the 5ml venous blood vacutainer after slaughtering of the animals and blood was allowed to clot for serum separation. The supernatant serum was transferred into 1.40 ml U-bottom tube and stored in a cooler-box and transported to ARC-OVI ELISA laboratory where they were stored at –20° C until use for serological analysis.

3.3.4 Sources of control samples

3.3.4.1 Negative control serum samples

Blood samples were collected from cattle that were raised under strict hygienic conditions for experimental use in ARC-OVI. The samples were sent to the ELISA laboratory in Parasite, Vector and Vector-borne Disease (PVVD) section, centrifuged and the resulting serum was tested for *Taenia* infections using HP10 AgELISA (Harrison *et al*. 1989). The confirmed *Taenia* negative sera were kept at –20°C until use in ELISA.

3.3.4.2 Blank samples

Blank samples were prepared from a blocking solution containing 450 ml of PBS, 5g of BSA and 250 µl of Tween 20.

3.3.4.3 Positive control serum samples

Positive serum samples were received from Dr LJS Harrison, University of Edinburgh, UK who is the collaborator in the project.

3.3.5 Serological analysis [(HP10 AgELISA (Harrison *et al.* (1989)] with some modifications

The procedure was as follows:

One Bicarbonate buffer capsule (C3401-Sigma) was dissolved into 100 ml of distilled water and 10 ml of the coating buffer (Appendix 5) was transferred into a mixing boat, followed by the addition of 20 µl of the prepared solution of 5mg/ml of McAb-HP10. The multichannel pipette was used to add 100 µl of the solution to each of the wells of a flat bottomed Immunlon 96-well ELISA plate. The plate was covered with plate cover to prevent evaporation and incubated overnight at 4°C. The wells were then washed twice with a washing solution

(Appendix 5) using an ELISA washer (Figure 3.7B) and 200 µl of a blocking solution (Appendix 5) was added to each of the wells and incubated at room temperature for 1 hour to block any non-reacted sites on the plate. The plates were then washed three times with washing solution. The 50 µl volume of undiluted sera was then added to each well, with each sample running in duplicate. The plate was incubated for 1 hour at 37°C in an incubator (Figure 3.7C), followed by emptying and washing three times. Biotinylated-McAb diluted 1:2,500 in PBS/BSA/Tween-20 was added at 100 µl/well, covered and incubated for 1 hour at 37°C followed again by washing three times. Streptavidin Peroxidase (Sigma S5512) conjugate diluted 1:10,000 (i.e. 0.1 µg/ml) in PBS/BSA/Tween-20 was added at 100 µl per well, covered and incubated for 1 hour at 37°C. After a further three washes 100 µl of 3, 3', 5, 5'-Tetramethylbenzidine substrate (Sigma T8665) substrate was then added to each well and incubated at room temperature for 30 minutes. The reaction was then stopped with 100 µl of 0.2 M H2SO4 per well and read at 450 nm on an ELISA plate reader (Figure 3.7D).

ELISA plates were routinely set up to include five positives, five negative and six diluent control wells (Figure 3.7 A), and each test sample was run in duplicate. The mean sample ODs minus the mean diluent ODs were corrected for any day to day variation using a correction factor determined by the formula:

Correction Factor = Mean P^0 - Mean N^0/ Mean P^t - Mean N^t

where P = positive control, N = negative control, 0 is the reference day and t is the test day.

ELISA results were rejected if the correction factor for any particular plate varied more than 10% from the reference day. Samples were run with different reagents and positive and negative controls; hence the negative cut off point was determined on a plate to plate basis using the formula: 2X + 3sd of negative controls, where X = the mean of the negative control and sd = standard deviation from the mean of the negative control.

Figure 3.7: Equipment used during the ELISA procedure: A 96 well plate with red, black and blue circles respectively indicating positive controls, negative diluent (A), ELISA plate washer (B), incubator (C) and ELISA plate

CHAPTER 4: RESULTS

4.1 Conventional PCR assay:

4.1.1 Optimisation of conventional PCR assay

4.1.1.1 Purity and concentration of positive control DNA samples

The Nanodrop results showed respective concentrations of 130 ng/µl, and 34 ng/µl with $A_{260}/_{280}$ ratio of 2.08, and 1.77 for *T. saginata* tapeworm and *T. solium* cysticercus.

A. HDP2 gene (Gonzalez *et al.* 2000)

The PTs7S35F1 and PTs7S35R1 primers amplified the expected 600 bp HDP2 in the *T. saginata* positive control sample in HotStar *Taq* PCR assay using the reagents at concentrations shown in Table 3.3 at the annealing temperature of 56.5°C, with the best amplification observed at the $MgCl_2$ concentrations of 3 Mm (Figure 4.1A). On the other hand, the PTs7S35F2 and PTs7S35R1 primers failed to amplify the 170 bp HDP2 gene from 2 µl of the *T. saginata* positive control sample in HotStar *Taq* PCR assay at MgCl2 concentrations varying from 1.5 to 5mM as indicated in Figure 4.1B.

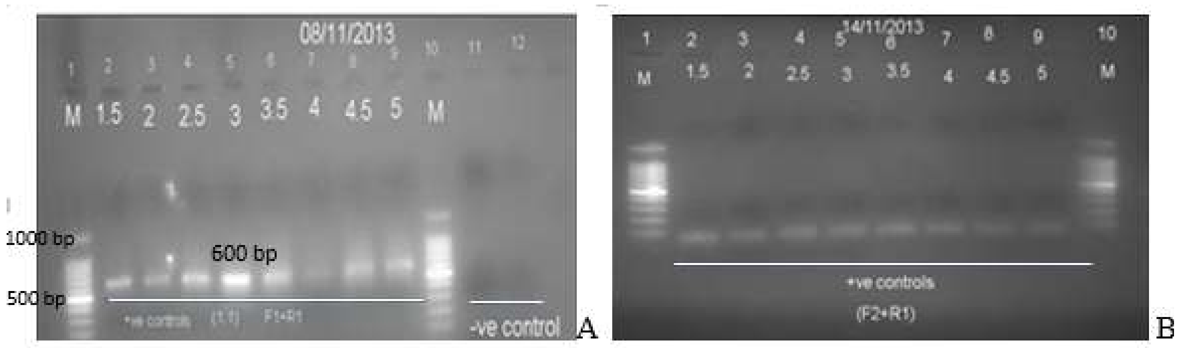


Figure 4.1: Detection of the 600 bp (A) and 170 bp (B) HDP2 gene in *T. saginata* positive control sample (tapeworm) at different $MgCl_2$ concentrations. M: DNA ladder (100 bp), negative control: distilled water.

B. Mitochondrial *cox*1 genes (Nkouawa *et al.* 2009)

The $MgCl_2$ concentrations of 3 mM used in HotStar *Taq* PCR assay resulted in the amplification of the target 238 bp *cox*1 gene in samples, S2 and S3 that were extracted by Roche and Qiagen kits respectively. However, the target gene was not detected in one of the field sample S1 which was extracted by Phenol chloroform extraction method in HotStar *Taq*

PCR assay (Figure 4.2A). On the other hand, the target gene was produced in all three samples in My *Taq* PCR assay even though faint DNA band was observed in sample S1 (Figure 4.2A).

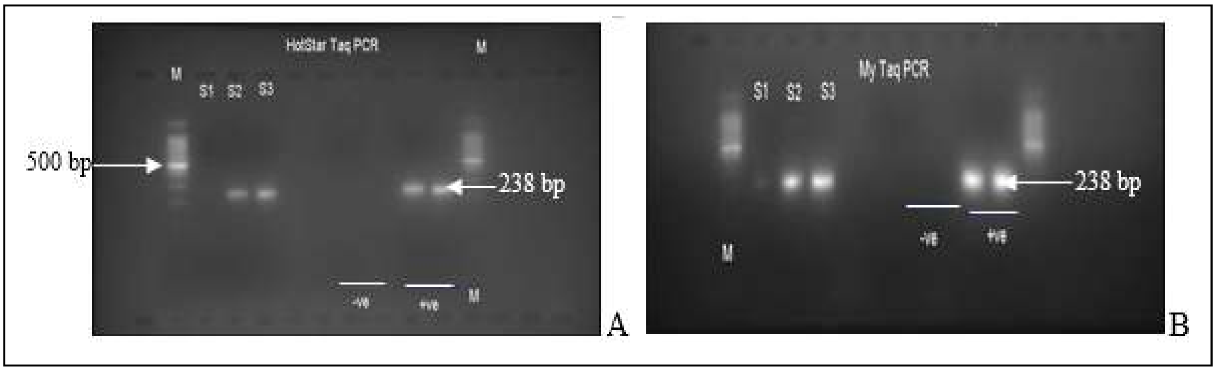


Figure 4.2: Detection of 238 bp *cox*1 gene in *T. saginata* tapeworm using HotStar *Taq* (A) and My *Taq* (B) PCR assays. M: DNA ladder (100 bp), positive control: *T. saginata*, negative control: distilled water.

The *T-sol* primers also amplified the expected *T. solium* 211 bp in 0.5 µl of the *T. solium* positive control and the field sample in My *Taq* PCR assay at the same annealing temperature of 58°C (Figure 4.3).

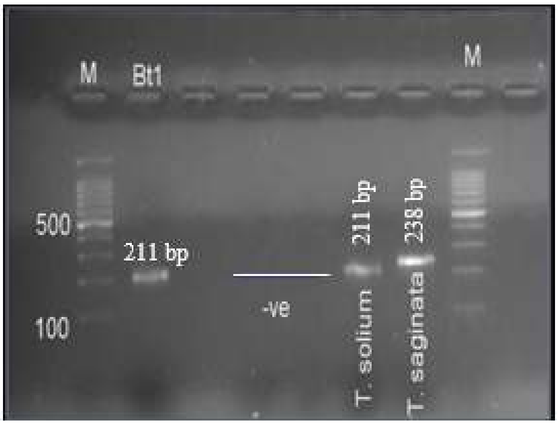


Figure 4.3: Detection of 211 bp *cox*1 gene in *T. solium* cysticerci using My *Taq* PCR assay. M: DNA ladder (100 bp), positive control: *T. solium*, negative control: *T. saginata*, negative control: distilled water.

Amplification of the *T. solium* 211 bp *cox*1 gene was successful in all tried different volumes of DNA extracted from porcine blood sample during optimisation of the detection of the target gene in the blood. However, bright DNA band was observed in 3 µl DNA template (Figure 4.4).

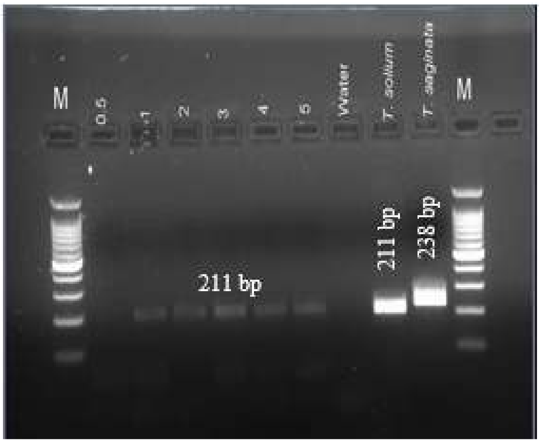


Figure 4.4: Detection of the 211 bp *cox*1 gene at different volumes ranging between 0.5 and 5 µl of DNA template of the porcine blood sample using My *Taq* PCR assay. M: DNA ladder (100 bp), positive control: *T. solium*, negative control *T. saginata*, negative control (distilled water).

4.1.1.2 Detection limit (sensitivity) of the PCR assay

The *T-sag cox*1 primers specific for *T. saginata* amplified the target gene at the lowest concentration of 0.0013 ng/ul (1 pg/µl) (Figure 4.5A). The *T-sol cox*1 primers amplified the *cox*1 gene at a lowest concentration of 0.34 ng/ul (100 pg/µl) from 10-fold serially diluted *T. solium* positive control sample (Figure 4.5B).

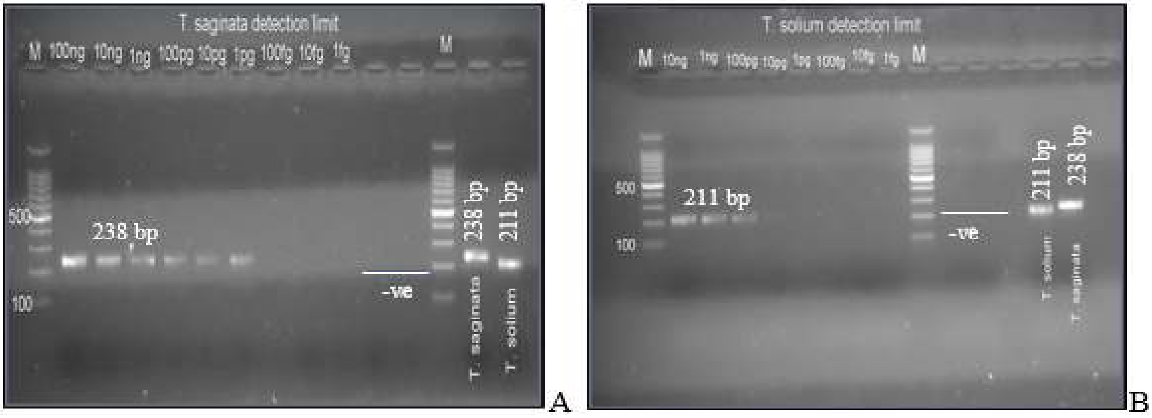


Figure 4.5: Detection limit of the *T. saginata* (A) and *T. solium* (B) PCR assays using *T. saginata* tapeworm and *T. solium* cysticercus DNAs positive controls.

4.1.2 Validation of conventional PCR assay

4.1.2.1 Study sites

Cattle and pigs blood samples were collected from 20 Free State abattoirs (13 low throughput and 7 high throughput) in all five districts whereas cyst samples were only collected in nine abattoirs (4 low throughput and 5 high throughput) in four districts (Figure 4.6). There were however instances where samples were collected more than once from the low throughput abattoirs which resulted in more than 49 blood samples being collected. Furthermore, abattoirs are identified by A – R throughout the study for confidentiality purposes.

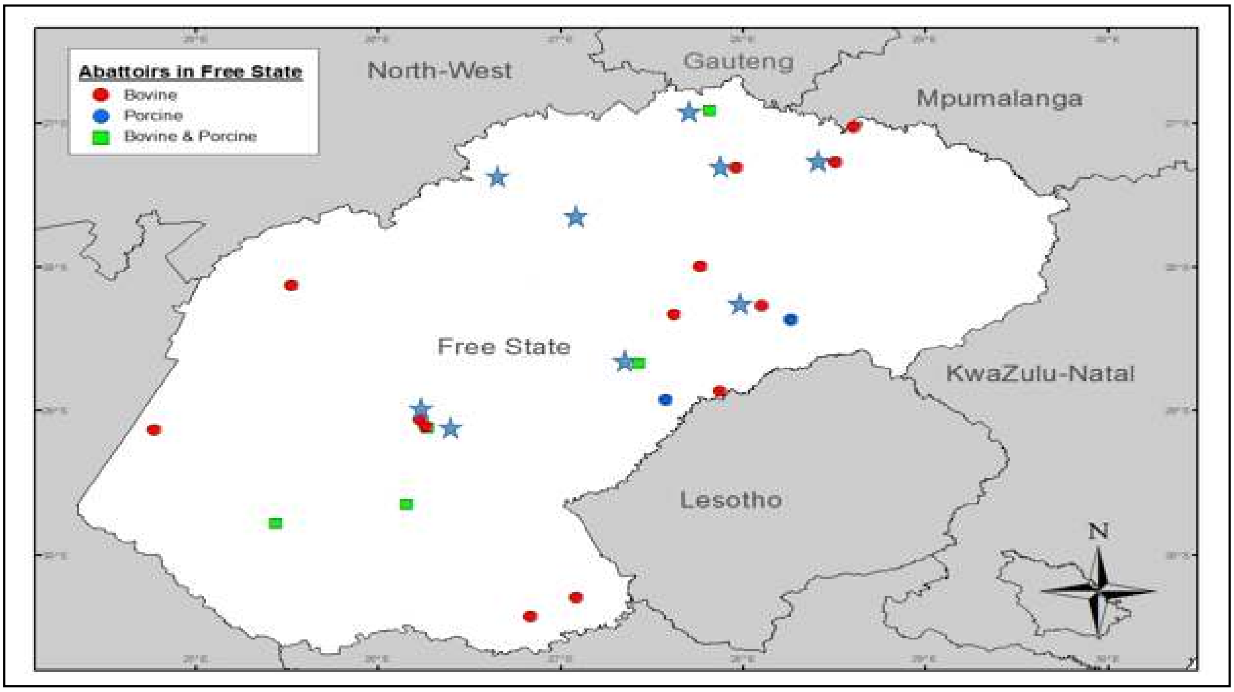


Figure 4.6: Map of Free State Province showing different sites of abattoirs visited. Collection areas for bovine blood samples, porcine blood samples, both bovine and porcine blood samples and cyst samples are respectively represented by 🔴 🔵 🟩 ★(Map drawn by R. Williams 2015).

4.1.2.2 Cysts as field samples

A total number of 73 cyst samples were detected and collected from four out of five districts of the Free State Province with 71 collected from bovine and 2 collected from porcine carcasses. The highest number of cyst samples was collected from bovine carcasses in Lejweleputswa district and only one cyst sample was collected in Thabo Mofutsanyana district. The two porcine cyst samples were each collected from Lejweleputswa and Thabo Mofutsanyana districts. The DNA concentration of most of the cyst samples collected had the spectrophotometer ratio between 1.7 and 1.9 except for 17 samples which had the ratio less than 1.7 as indicated in Table 4.1 and none of the samples had ratio of greater than 2.

All collected cyst samples identified as *T. saginata* and *T. solium* cysticerci during meat inspection tested positive and showed the overall PCR detection efficiency of 100% (Figure 4.7). However, initially only 87% (62/71) of samples identified as *T. saginata* cysticerci tested positive, and the remaining nine samples later showed weak positive results after being repeatedly tested.

Table 4.1: DNA spectrophotometer results of cyst samples collected from Free State abattoirs.

Extraction method	**Sample ID**	**[ng/µl]**	A_{260}/A_{280}
Motheo District:			
C abattoir	1	168.97	1.81
	2	145.32	1.85
	3	26.86	1.79
	4	168.83	1.89
	5	17.66	1.83
	6	35.74	1.54
	7	159.48	1.77
	8	71.85	1.65
	9	61.96	1.81
	10	61.96	1.74
	11	21.74	1.80
	12	36.37	1.75
	13	262.74	1.72
	14	482.17	1.89
B abattoir	1	50.28	1.78
	2	51.96	1.87
	3	58.10	1.16
	4	44.11	1.53
Lejweleputswa District			
V abattoir	1 (porcine)	34.47	1.72
	2	22.72	1.84
	3	13.96	1.58
	4	50.40	1.32
	5	18.42	1.88
W abattoir	1	60.26	1.80
	2	51.03	1.84
	3	120.43	1.79
	4	86.92	1.62
	5	45.78	1.39
	6	122.01	1.80
	7	148.18	1.87
	8	22.45	1.84
	9	63.22	1.73
	10	143.39	1.78
	11	5.64	1.03
	12	10.17	1.23
	13	128.01	1.80
	14	110.49	1.84
	15	88.34	1.80
	16	67.32	1.76
	17	38.43	1.60

	18	45.73	1.82
	19	105.33	1.84
	20	110.98	1.83
	21	108.43	1.87
	22	124.54	1.77
	23	163.03	1.87
	24	143.09	1.56
	25	164.68	1.88
	26	24.54	1.78
	27	6.04	1.35
	28	78.39	1.84
	29	64.03	1.79
	30	10.14	1.68
Fezile Dabi District:			
U abattoir	1	45.69	1.88
	2	5.43	1.63
	3	70.32	1.61
	4	10.65	1.39
	5	53.81	1.74
O abattoir	1	57.32	1.42
Q abattoir	1	11.89	1.32
	2	24.23	1.46
N abattoir	1	101.04	1.84
	2	56.72	1.73
	3	63.28	1.80
	4	38.25	1.65
	5	85.43	1.83
	6	84.68	1.88
	7	78.08	1.79
	8	49.23	1.80
	9	93.26	1.84
	10	28.34	1.38
Thabo Mofutsanyana District:			
D abattoir	1	65.38	1.72
	2 (porcine)	58.01	1.87

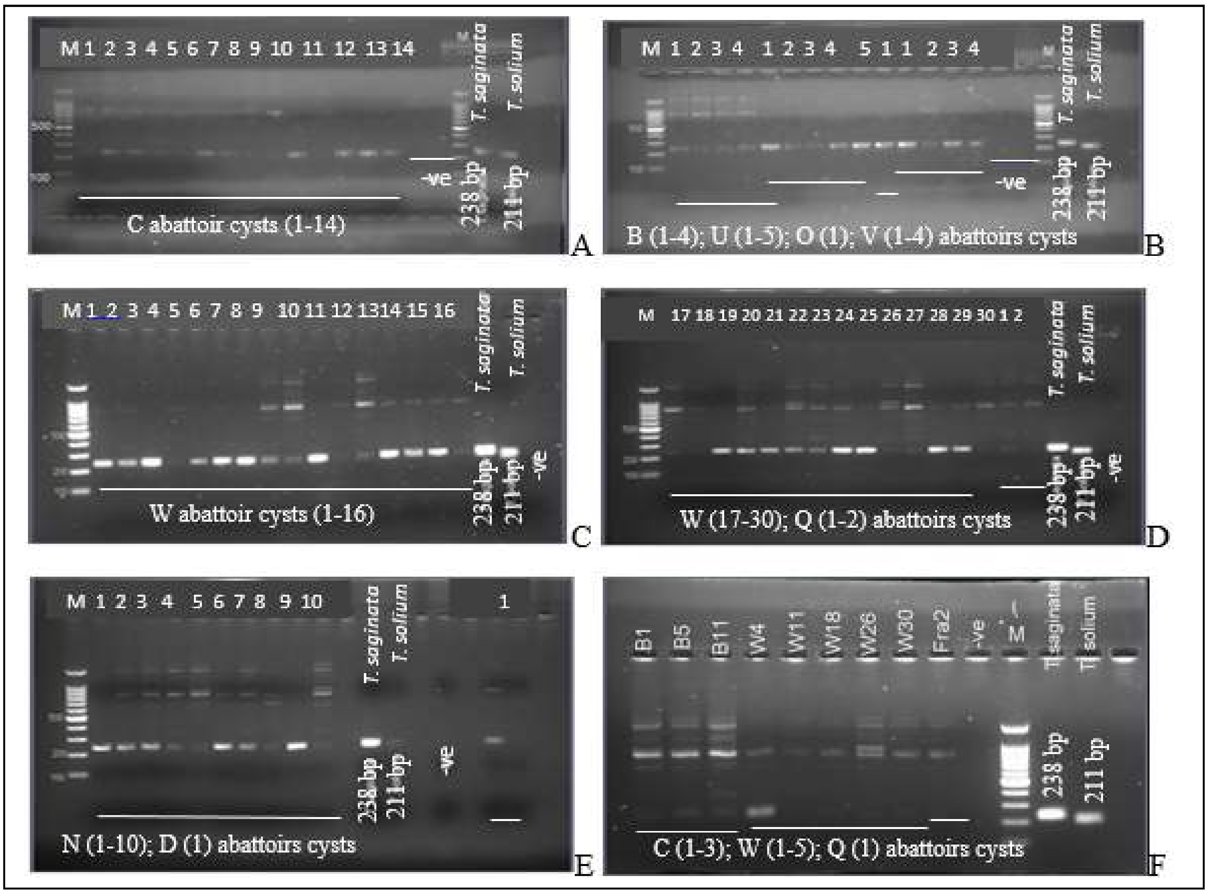


Figure 4.7: Detection of the 238 bp *T. saginata cox*1 gene (A-F) in suspected field bovine cyst samples screened with optimised My *Taq* PCR assay. M: DNA ladder (100 bp), positive control: *T. saginata*, negative control *T. solium*, negative control (distilled water).

Two porcine cyst samples tested positive with the optimised assay producing the expected 211 bp *T. solium cox*1 gene (Figure 4.8).

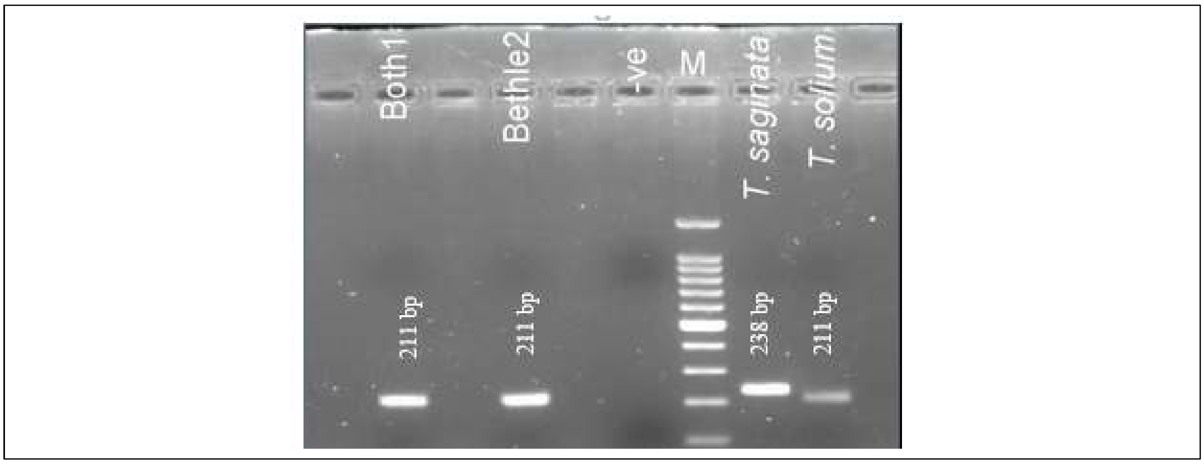


Figure 4.8: Detection of the 211 bp *T. solium cox*1 gene in two porcine cysticerci samples collected in V abattoir (Both1) and D abattoir (Bethle2). M: DNA ladder (100 bp), positive control: *T. solium*, negative control: *T. saginata*, negative control: distilled water.

4.1.2.3 Blood as field samples

Pooled bovine and porcine blood samples respectively tested positive for detection of target 238 bp *T. saginata cox*1 and 211 bp *T. solium cox*1 genes, although very faint DNA bands were observed on porcine blood results (Figure 4.9). The test of the pooled samples from Sasolburg abattoirs tested negative.

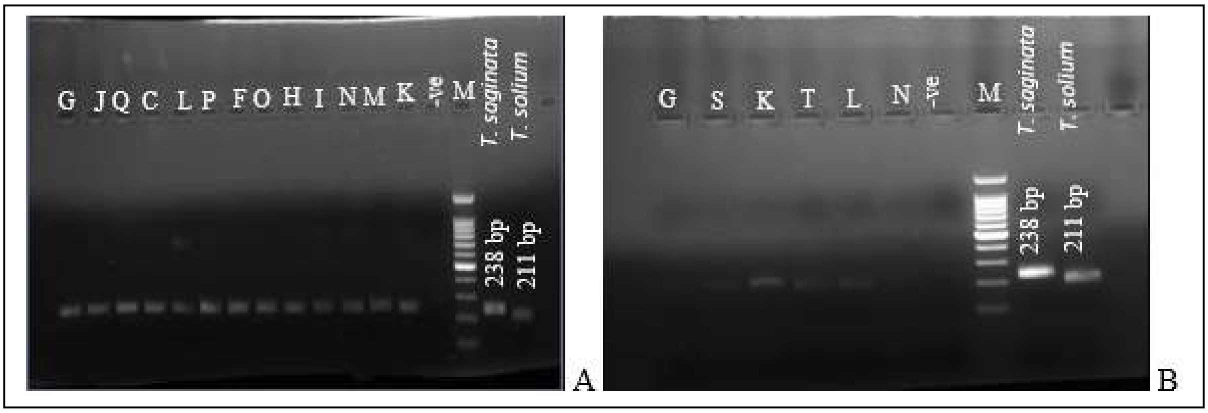


Figure 4.9: Detection of the *T. saginata* (A) and *T. solium* (B) *cox*1 genes in pooled bovine and porcine blood samples. M: DNA ladder (100 bp), positive control: *T. saginata*, positive control *T. solium*, negative control: distilled water.

The gene of interest at a size of 238 bp was detected in 577 individual bovine blood samples as shown in the first 21 abattoir J from Xhariep district, Figure 4.10A. The target 211 bp *T. solium cox*1 gene was not observed in all 233 porcine blood samples when tested individually as shown in the first eight samples of abattoir C from Motheo district, Figure 4.10B.

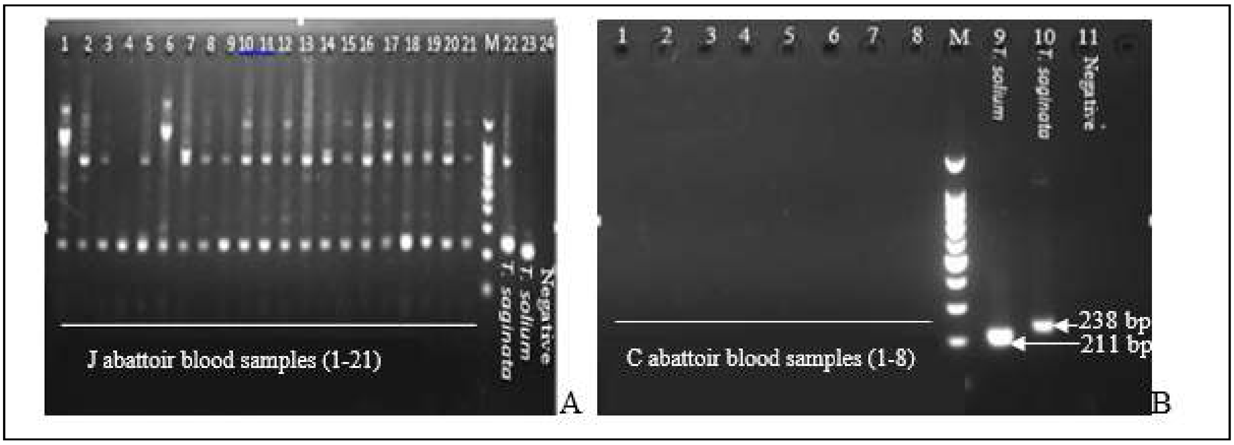


Figure 4.10: Detection of 238 bp *T. saginata cox*1 (A) and 211 bp *T. solium cox*1 (B) genes in bovine and porcine blood samples. A: 1-21: Bovine blood samples, 22: *T. saginata* positive control, 23*: T. solium* negative control, 24: negative control (distilled water). B: 1-8: Porcine

blood samples 9: *T. solium* positive control, 10: *T. saginata* negative control, 11: negative control (distilled water), M: DNA ladder (100 bp).

The *T. saginata* PCR showed a high detection efficiency as 94% (577/614) of blood samples tested positive in Free State Province with the highest efficiency recorded in Fezile Dabi district (99%) and the lowest (85%) recorded in Lejweleputswa district. A 0% detection efficiency was recorded in *T. solium* PCR as none of the 233 screened porcine blood samples tested positive with the optimised PCR assay. Detection efficiencies of *T. saginata* and *T. solium* PCR assays tested on cattle and pigs brought for slaughter in the Free State abattoirs are depicted in Tables 4.2 and 4.3.

Table 4.2: PCR efficiency in detecting bovine cysticercosis in cattle brought for slaughter at abattoirs in five districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	Low/high throughput	PCR positive results
Motheo		**96% (70/73)**
A	Low	86% (14/16)
B	Low	86% (6/7)
C	High	100% (50/50)
Thabo Mofutsanyana		**96% (157/164)**
D	Low	89% (17/19)
E	High	90% (45/50)
F	Low	100% (36/36)
G	Low	100% (26/26)
H	High	100% (33/33)
Xhariep		**89% (187/209)**
I	High	96% (48/50)
J	Low	100% (50/50)
K	Low	100% (35/35)
L	Low	61% (30/49)
M	Low	96% (24/25)
Fezile Dabi		**99% (141/142)**

N	High	100% (30/30)
O	High	100% (50/50)
P	Low	92% (11/12)
Q	Low	100% (50/50)
Lejweleputswa		**85% (22/26)**
R	Low	85% (22/26)
Total	**12 Low-; 6 High throughput**	**94% (577/614)**

Table 4.3: PCR efficiency in detecting porcine cysticercosis in pigs brought for slaughter at abattoirs in four districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	**Low/high throughput**	**PCR positive results**
Motheo		**0% (0/8)**
C	High	0% (0/8)
Thabo Mofutsanyana		**0% (0/98)**
G	Low	0% (0/28)
S	Low	0% (0/20)
T	High	0% (0/50)
Xhariep		**0% (0/101)**
K	Low	0% (0/50)
L	Low	0% (0/51)
Fezile Dabi		**0% (0/26)**
N	Low	0% (0/26)
Total	**5 Low-; 2 High throughput**	**0% (0/233)**

4.2 *Taq*Man real-time PCR assay

4.2.1 Optimisation of annealing temperature of primers and probes

Only *T. solium* was used for determination of the optimal annealing temperature for detection of the *cox*1 gene. Since the target gene of *T. saginata* and *T. solium* are both mitochondrial *cox*1 gene therefore conditions used for amplification of *T. solium cox*1 gene also applied for *T. saginata* even though the target genes of the two species differ in size. The flourescence signals of the amplification curves picked up at similar Ct values when different annealing temperatures of the *T. solium* primers and probes were tested (Figures 4.11). Annealing temperatures of 55ºC and 57ºC produced the Ct value of 18.5 while 56ºC and 58ºC produced the Ct value of 19.5.

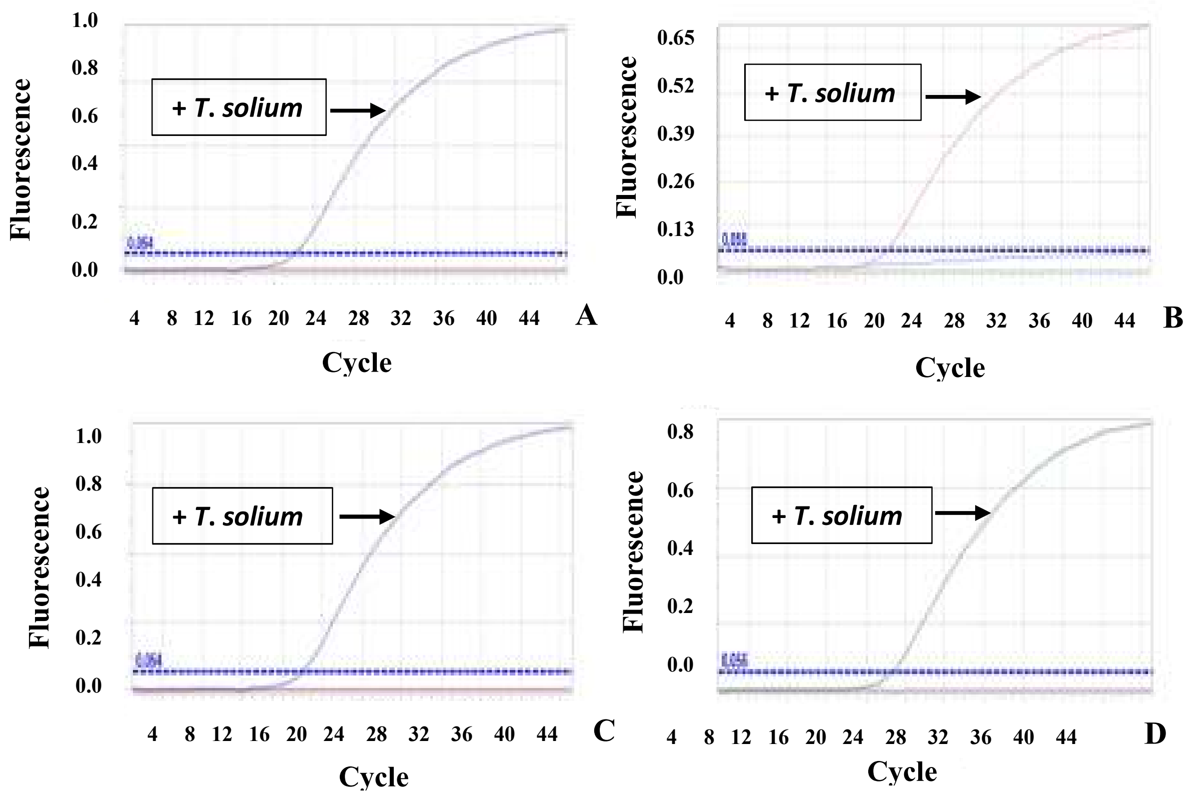
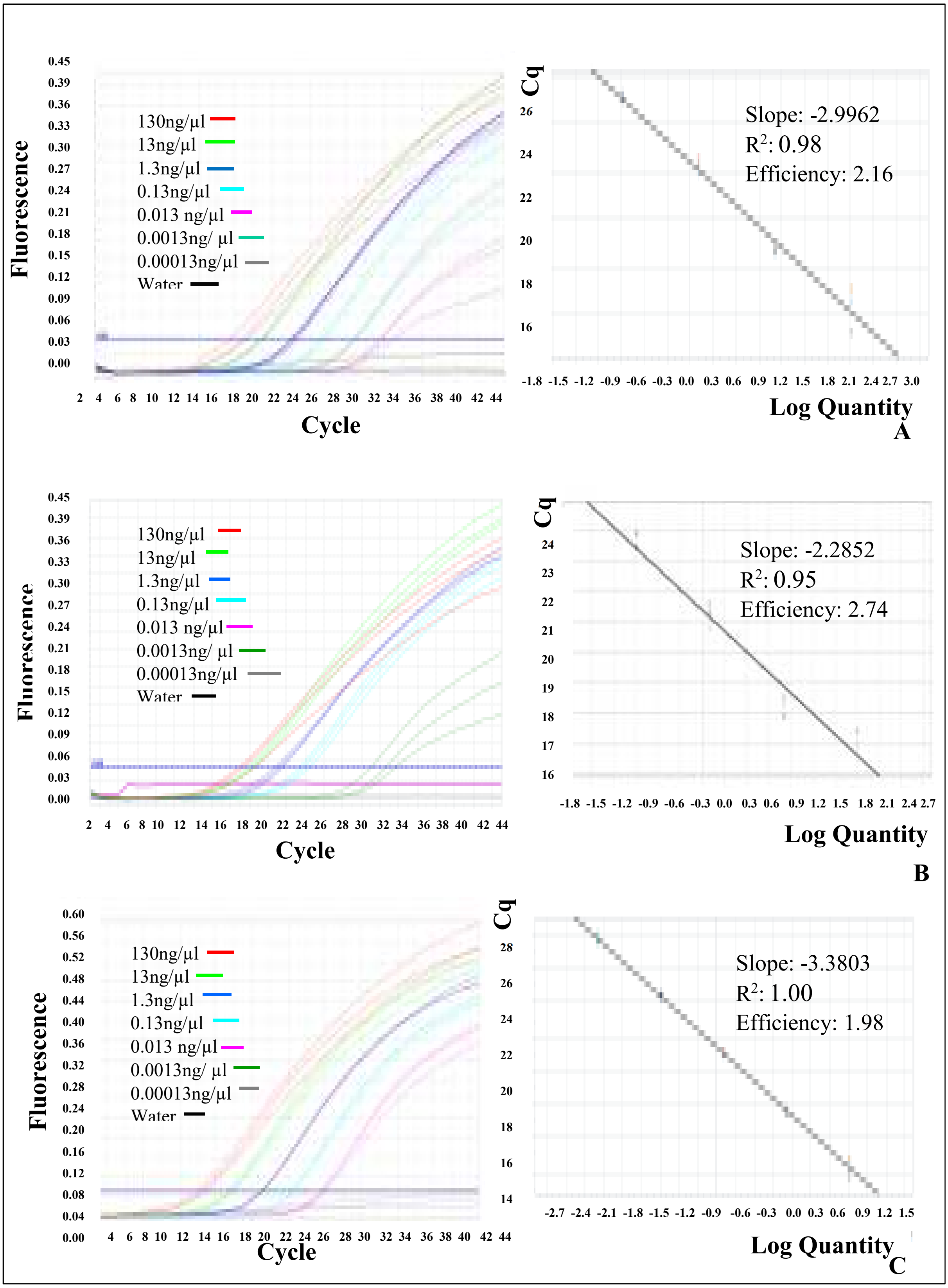


Figure 4.11: Different annealing temperatures, 55ºC (A), 56ºC (B), 57ºC (C) and 58ºC (C) for primers and probes tested for detection of the *T. solium cox*1 gene.

4.2.1.1 Sensitivity of *T. saginata* and *T. solium* real-time PCR assays

4.2.1.1.1 *Taenia saginata* real-time PCR assay

The accuracy of the *T. saginata* real-time PCR was determined through triplicates of serial dilution containing DNA of the *T. saginata* positive control sample. Five experiments produced standard curves of different slopes and different PCR efficiencies (Figure 4.12) during the determination for the detection limit determination of *T. saginata* real-time PCR assay. The target *T. saginata cox*1 gene was amplified at the lowest concentration of 0.0013ng/µl corresponding to the Ct values of 30.62, 30.02 and 31.40 as indicated in Table 4.4. Out of five experiments performed, the third experiment was regarded as the best optimal for amplification of experiments performed, the third experiment was regarded as the best optimal for amplification of the target gene for *T. saginata*.


Fluorescence
0.45
0.39
0.36
0.33
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
130ng/µl
13ng/µl
1.3ng/µl
0.13ng/µl
0.013 ng/µl
0.0013ng/ µl
0.00013ng/µl
Water
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
Cq
26
24
22
20
18
16
Slope: -2.9962
R²: 0.98
Efficiency: 2.16
-1.8 -1.5 -1.2 -0.9 -0.6 -0.3 0.0 0.3 0.6 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0
Log Quantity
A
Fluorescence
0.45
0.39
0.36
0.33
0.30
0.27
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
130ng/µl
13ng/µl
1.3ng/µl
0.13ng/µl
0.013 ng/µl
0.0013ng/ µl
0.00013ng/µl
Water
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
Cq
24
23
22
21
20
19
18
17
16
Slope: -2.2852
R²: 0.95
Efficiency: 2.74
-1.8 -1.5 -1.2 -0.9 -0.6 -0.3 0.0 0.3 0.6 0.9 1.2 1.5 1.8 2.1 2.4 2.7
Log Quantity
B
Fluorescence
0.60
0.56
0.52
0.48
0.44
0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
130ng/µl
13ng/µl
1.3ng/µl
0.13ng/µl
0.013 ng/µl
0.0013ng/ µl
0.00013ng/µl
Water
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
Cq
28
26
24
22
20
18
16
14
Slope: -3.3803
R²: 1.00
Efficiency: 1.98
-2.7 -2.4 -2.1 -1.8 -1.5 -1.2 -0.9 -0.6 -0.3 0.0 0.3 0.6 0.9 1.2 1.5
Log Quantity
C

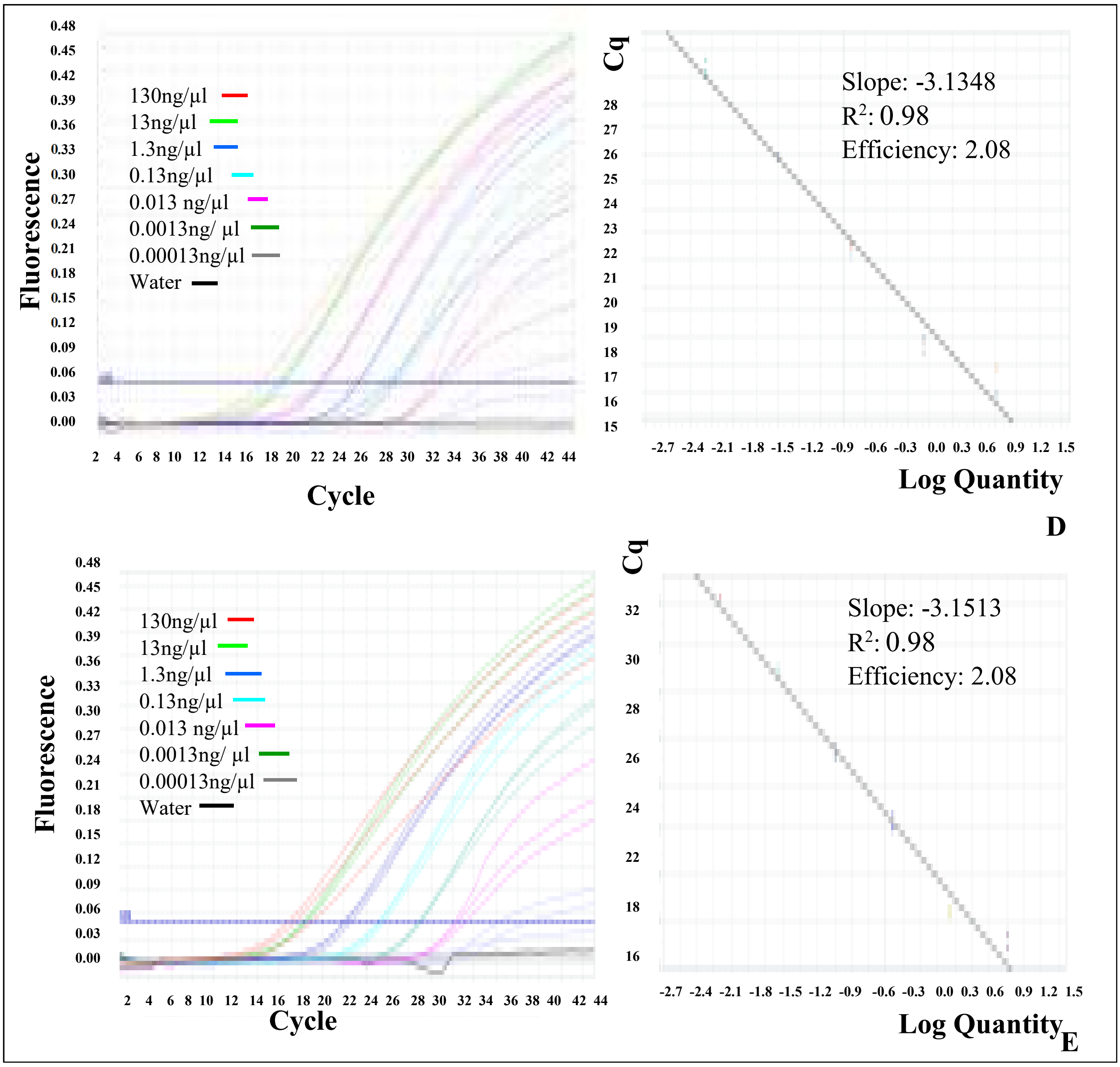


Figure 4.12: Amplification and standard curves of sensitivity of *T. saginata* real-time PCR assay with sample dilutions in triplicates.

Table 4.4: Threshold cycle (Ct) values produced in 5 performed experiments from dilutions of *T. saginata* positive control sample tested in triplicates**.**

	Expt 1		**Expt 2**		**Expt 3**		**Expt 4**		**Expt 5**	
Dilutions (ng/µl)	Ct	Call	Ct	Call	Ct	Call	Ct	Call	Ct	Call
130	16.56	+	16.92	+	16.76	+	15.78	+	16.84	+
130	15.27	+	17.37	+	15.96	+	15.85	+	17.43	+
130	17.07	+	0.00	+	16.87	+	16.85	+	0.00	-
13	18.69	+	17.88	+	17.39	+	17.90	+	18.18	+
13	18.54	+	18.26	+	17.46	+	18.02	+	18.38	+
13	18.81	+	18.47	+	17.75	+	17.43	+	18.55	+
1.3	22.55	+	21.56	+	21.06	+	21.49	+	22.01	+
1.3	22.26	+	21.26	+	21.04	+	21.69	+	22.65	+
1.3	21.93	+	20.78	+	20.86	+	21.13	+	22.13	+
0.13	25.00	+	23.41	+	24.33	+	0.00	-	25.11	+
0.13	0.00	-	23.88	+	24.32	+	24.93	+	25.29	+
0.13	25.22	+	0.00	-	24.50	+	24.90	+	25.67	+
0.013	0.00	-	0.00	-	27.68	+	28.05	+	29.08	+
0.013	0.00	-	0.00	-	27.58	+	28.15	+	28.82	+
0.013	0.00	-	0.00	-	27.99	+	28.64	+	0.00	-
0.0013	0.00	-	0.00	-	30.62	+	31.41	+	32.17	+
0.0013	0.00	-	0.00	-	30.02	+	0.00	-	0.00	-
0.0013	0.00	-	0.00	-	31.40	+	0.00	-	0.00	-
0.00013	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
0.00013	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
0.00013	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
Water	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
Water	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
Water	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-

4.2.1.1.2 *Taenia solium* real-time PCR assay

The amplification curves in the *T. solium* real-time PCR assay were observed up to the dilution concentration of 0.0034 ng/µl. The sensitivity reactions of the assay produced the standard curve with the Slope:-3.3730, Efficiency: 1.98, Error: 0.76 and R^2: 0.98 (Figure 4.13). There were no C values produced after the concentration of 0.0034 ng/µl (Table 4.5).

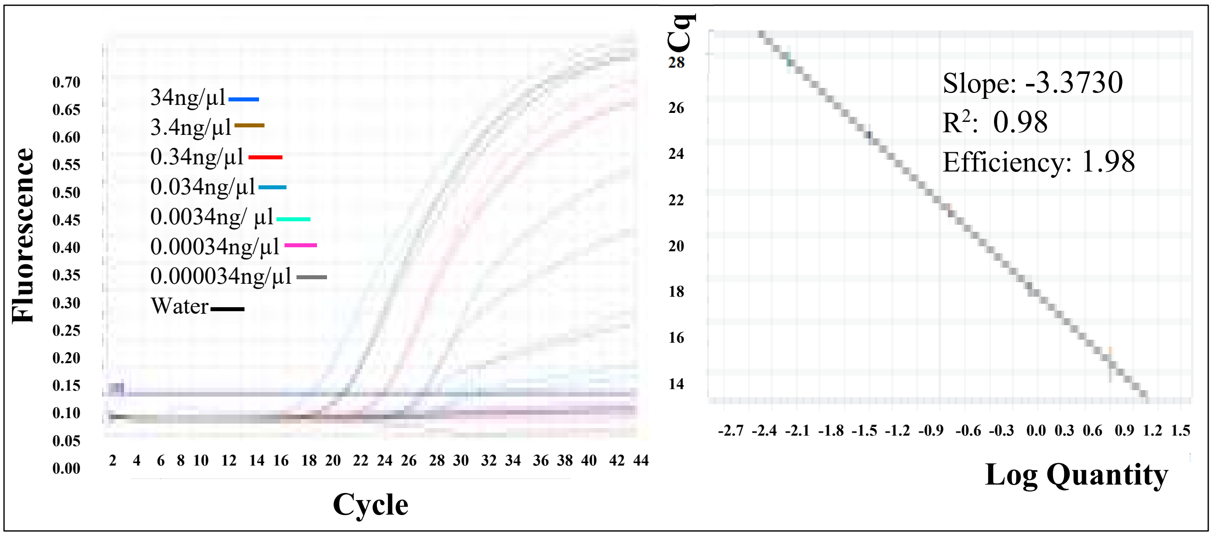


Figure 4.13: Amplification and standard curves of sensitivity of *T. solium* real-time PCR assay with sample dilutions in triplicates.

Table 4.5: Threshold cycle (Ct) values produced in the performed experiment from dilutions of *T. solium* positive control sample tested in triplicates.

Dilutions (ng/µl)	Ct	Call
34	17.93	+
34	17.99	+
34	18.09	+
3.4	20.53	+
3.4	20.58	+
3.4	20.73	+
0.34	23.83	+
0.34	24.04	+
0.34	23.96	+
0.034	28.38	+
0.034	27.64	+
0.034	28.91	+
0.0034	33.25	+
0.0034	30.09	+
0.0034	31.11	+
0.00034	0.00	-
0.00034	0.00	-
0.00034	0.00	-
0.000034	0.00	-
0.000034	0.00	-
0.000034	0.00	-
Water	0.00	-
Water	0.00	-
Water	0.00	-

4.2.1.2 Specificities of *T. saginata* and *T. solium* real-time PCR assays

4.2.1.2.1 *Taenia saginata* real-time PCR assay

Only the *T. saginata* positive control DNA was amplified in the specificity test against other related *Taenia* species and host DNA (Figure 4.14). The fluorescence signals of the amplification curves of the three *T. saginata* positive controls picked up at a range of between 16 and 17 cycles.

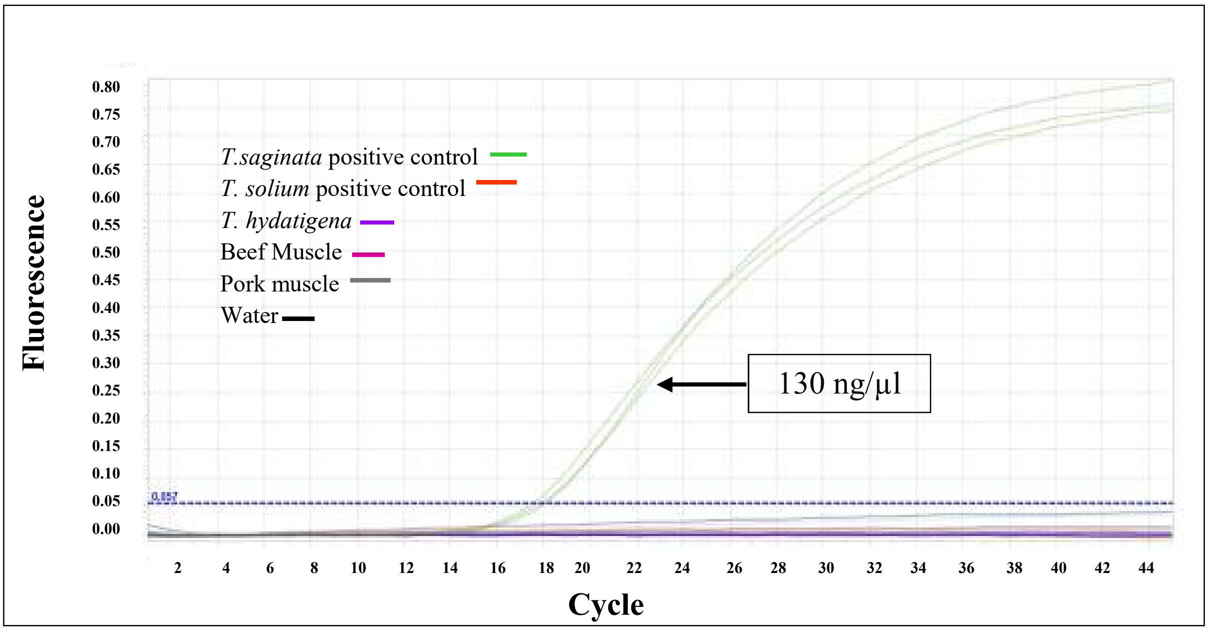


Figure 4.14: Amplification curves showing the specificity of *T. saginata* real-time PCR assay.

4.2.1.2.2 *Taenia solium* real-time PCR assay

Taenia solium positive control samples produced the amplification curves (Figure 4.15) with the Ct values of 16.86, 17.15 and 16.94 for the *T. solium* real-time PCR assay specificity test. The amplification curves were not produced in other *Taenia* species, *T. saginata* and *T. hydatigena* samples and host muscles of pork and beef.

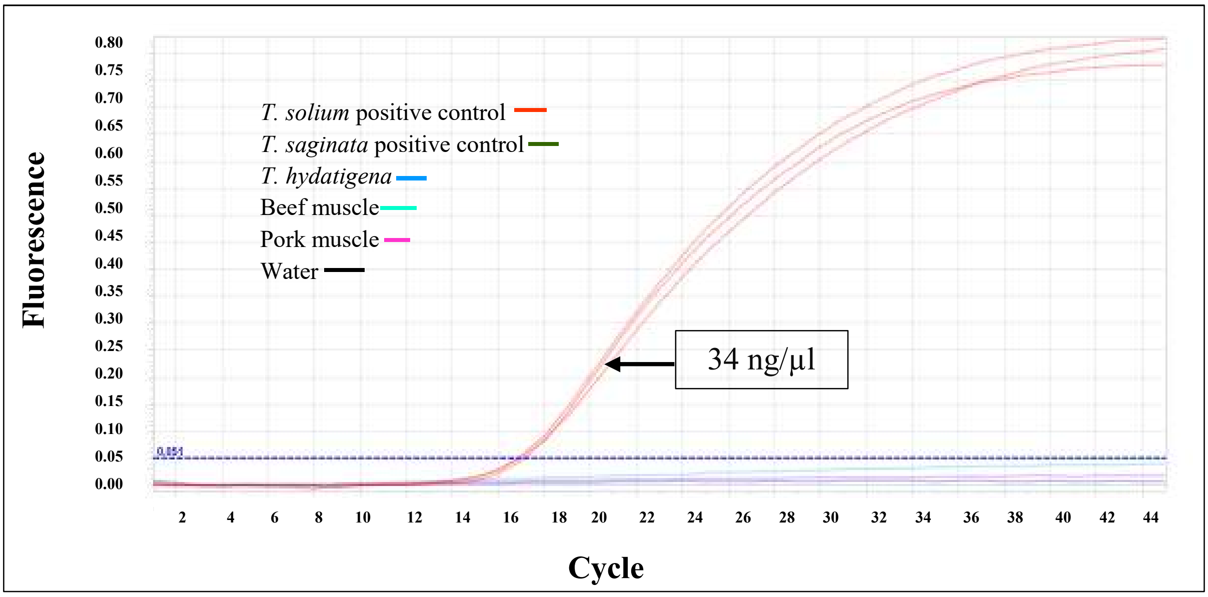


Figure 4.15: Amplification curves showing the specificity of *T. solium* real-time PCR assay.

4.2.2 Validation of real-time PCR assays using field samples

4.2.2.1 Cysts as field samples

A total of 71 bovine cyst samples were collected from bovine carcasses in four districts of the Free State Province, Motheo (n = 18), Lejweleputswa (34), Fezile Dabi (n = 18) and Thabo Mofutsanyana (n = 1). The highest detection efficiency in bovine samples was detected in Lejweleputswa district with 82.35% (28/34), and the lowest was in Thabo Mofutsanyana with 0% (0/1) as indicated in Table 4.6. The optimised *T. saginata* real-time PCR assays confirmed 63.38% (45/71) cyst samples identified as *T. saginata* cysticerci (Table 4.6). On the other hand 2 samples that were collected from pigs in two low throughput abattoirs were also confirmed positive producing detection efficiency of 100% (2/2) with *T. solium* assay.

Table 4.6: Real-time PCR efficiency in confirming cyst samples identified as *T. saginata* cysticerci at abattoirs in four districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	Low/high throughput	qPCR positive results
Motheo		**33% (6/18)**
B	Low	50% (2/4)
C	High	29% (4/14)
Thabo Mofutsanyana		**0% (0/1)**
D	Low	0% (0/1
Fezile Dabi		**61% (11/18)**
N	High	60% (6/10)
O	High	100% (1/1)
Q	Low	0% (0/2)
U	High	80% (4/5)
Lejweleputswa		**82% (28/34)**
V	Low	75% (3/4)
W	High	83% (25/30)
Total	**4 Low-; 5 High throughput**	**63% (45/71)**

The amplification curves of all bovine samples fluoresced later at cycles between 19.92 and 43.80 except for one sample from W abattoir which fluoresced earlier at cycle 15 which correspond with that of the positive control sample (Figure 4.16A). The two porcine cyst samples collected from Free State tested positive with Ct value of 15.95 one from V abattoir and the second one from D abattoir fluoresced at cycle 22.41 (Figure 4.16B).

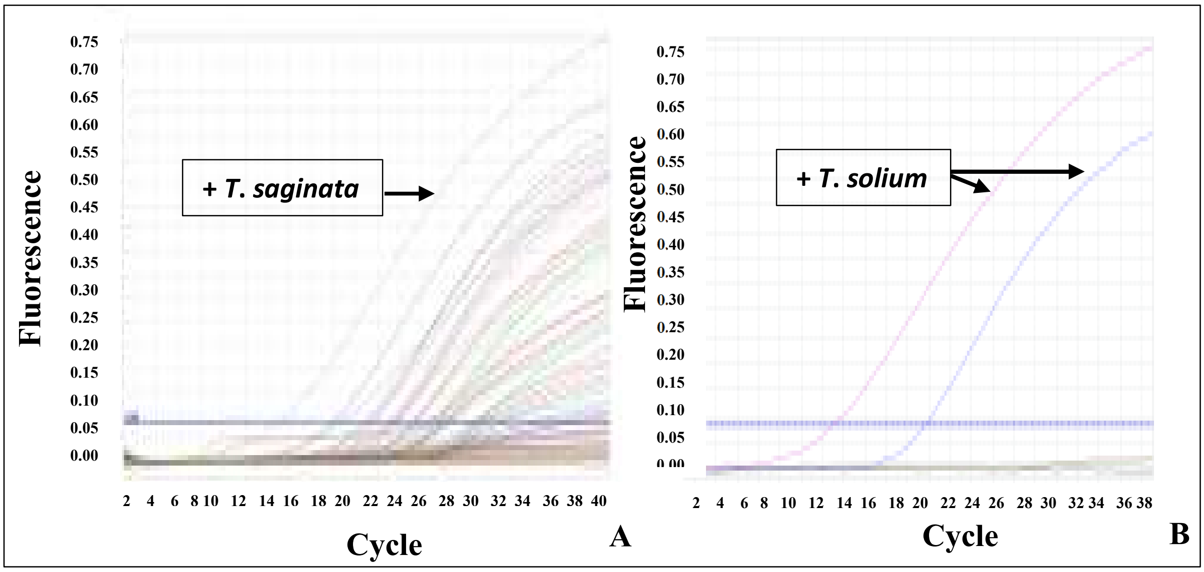


Figure 4.16: Amplification curves of bovine (A) and porcine (B) cyst samples from Free State abattoirs screened for *T. saginata* and *T. solium cox*1 genes with developed real-time PCR assays.

4.2.2.2 Blood as field samples

A total of 496 bovine blood samples collected from the five Free State districts were screened with the new developed assay, Motheo (n = 73), Fezile Dabi (n = 142), Thabo Mofutsanyana (n = 164), Xhariep (n = 209) and Lejweleputswa (n = 26). The number of bovine blood samples collected from various abattoirs in each district is indicated in Table 4.7. The *T. saginata* real-time PCR assay detected 75% (458/614) positives and the highest detection efficiency in bovine blood samples was in Xhariep district with 83% (174/209), and Lejweleputswa district with 42% (11/26) (Table 4.7). The amplification curves of all bovine field samples fluoresced after the *T. saginata* positive control sample (Figure 4.17).

Table 4.7: Real-time PCR efficiency in detecting bovine cysticercosis in cattle brought for slaughter at abattoirs in five districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	**Low/high throughput**	**PCR positive results**
Motheo		**75% (55/73)**
A	Low	56.25% (9/16)
B	Low	14.28% (1/7)
C	High	90% (45/50)
Thabo Mofutsanyana		**66% (108/164)**
D	Low	57.89% (11/19)
E	High	24% (12/50)
F	Low	94.44% (34/36)
G	Low	76.92% (20/26)
H	High	93.93% (31/33)
Xhariep		**83% (174/209)**
I	High	78% (39/50)
J	Low	90% (45/50)
K	Low	65.71% (23/35)
L	Low	57.14% (28/49)
M	Low	78% (39/25)
Fezile Dabi		**77% (110/142)**
N	High	73.33% (22/30)
O	High	84% (42/50)
P	Low	91.6% (11/12)
Q	Low	70% (35/50)
Lejweleputswa		**42% (11/26)**
R	Low	42% (11/26)
Total	**12 Low-; 6 High throughput**	**75% (458/614)**

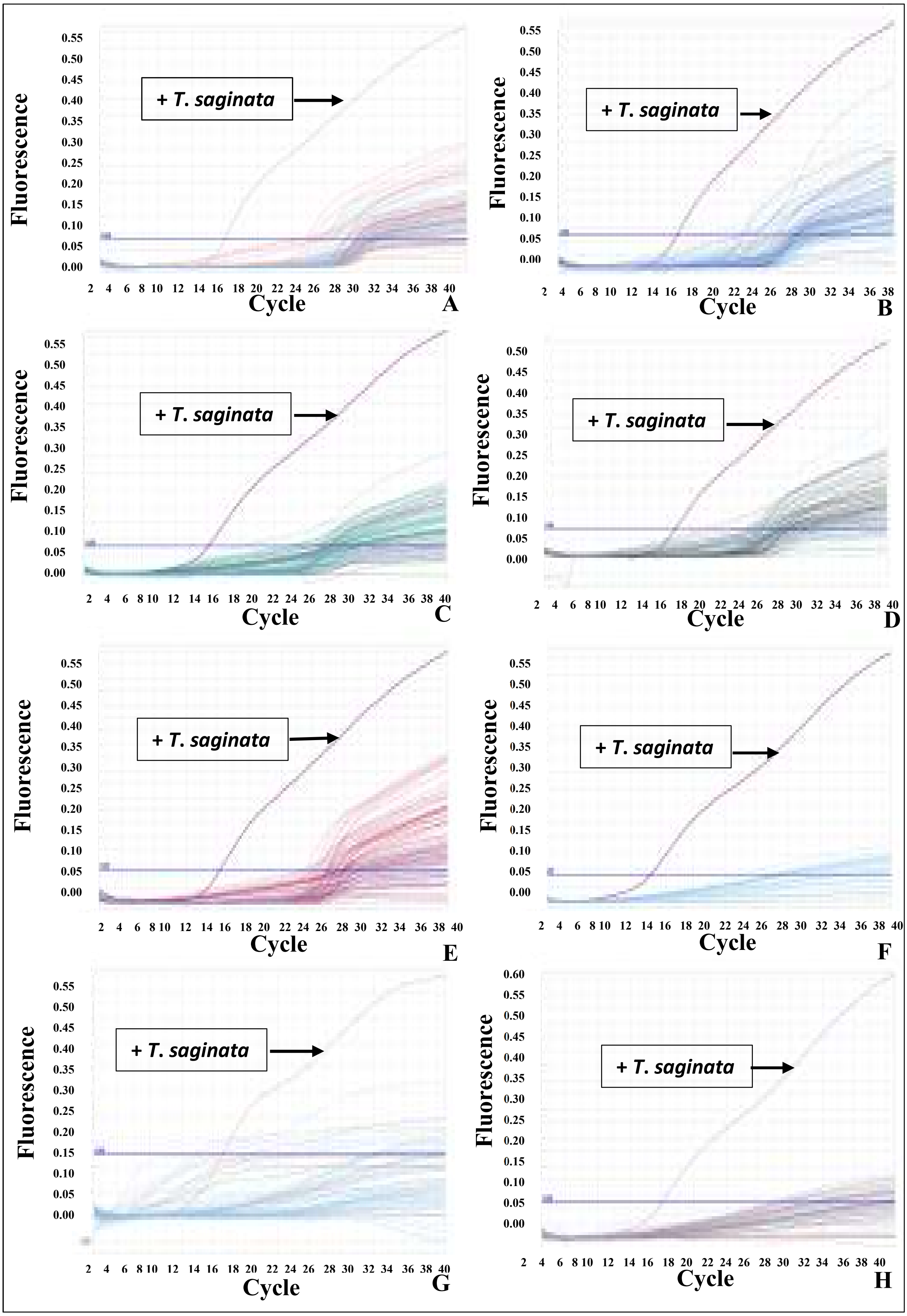


Figure 4.17: Amplification curves results of screened bovine blood samples (A-H) from Free State abattoirs using the newly developed *T. saginata* real-time PCR assay.

A total of 233 porcine blood samples collected from two districts of the Free State Province were also screened, Fezile Dabi (n = 26), Thabo Mofutsanyana (n = 98) , Xhariep (n = 101) and Motheo (n = 8). A total of 76 out of 233 porcine blood samples tested positive when screened with the developed real-time PCR assay. The assay yielded the overall detection efficiency of 33% with Fezile Dabi district showing the highest prevalence (77%) and Motheo district with the lowest prevalence of 12.5% (Table 4.8). Also with the porcine sample, the amplification curves of the field samples fluoresced later than the *T. solium* positive control sample at cycles between 27.19 and 43 (Figure 4.18). The assay will be used as described in Appendix 4 even though its sensitivity was affected by the degeneration of the cysts which can be solved by proper storage of the samples.

Table 4.8: Real-time PCR efficiency in detecting porcine cysticercosis in pigs brought for slaughter at abattoirs in four districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	Low/high throughput	PCR positive results
Motheo		**12.5% (1/8)**
C	High	12.5% (1/8)
Thabo Mofutsanyana		**26.53% (26/98)**
G	Low	39.28% (11/28)
S	Low	30% (6/20)
T	High	18% (9/50)
Xhariep		**29% (29/101)**
K	Low	34% (17/50)
L	Low	23.52% (12/51)
Fezile Dabi		**77% (20/26)**
N	Low	76.92% (20/26)
Total	**5 Low-; 2 High throughput**	**33% (76/233)**

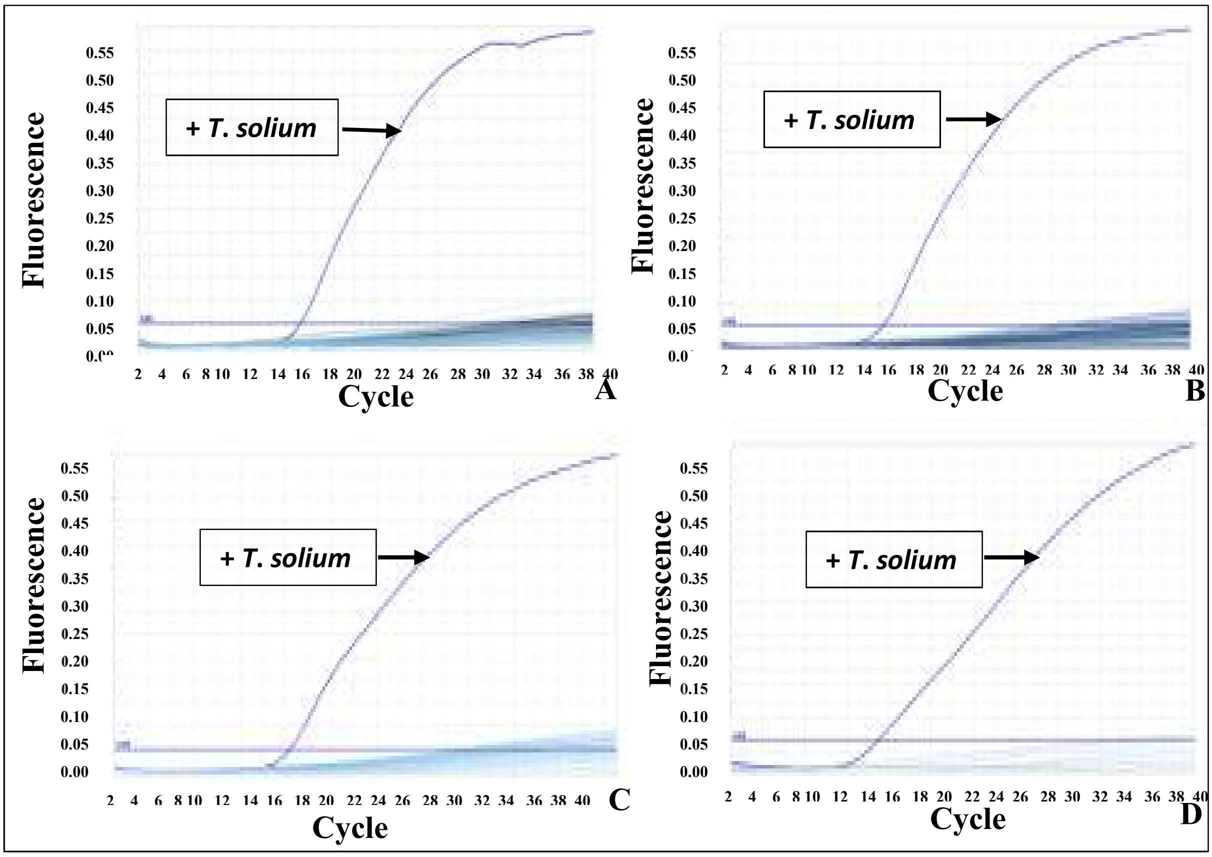


Figure 4.18: Amplification curves results of screened porcine blood samples (A-D) from Free State abattoirs using the newly developed *T. solium* real-time PCR assay.

4.3 HP10 (McAb) AgELISA assay

4.3.1 Detection of *Taenia* infection in cattle blood samples using HP10 (McAb) AgELISA assay

Serological analysis was done on 320 bovine samples in which 73 were from Motheo district, 69 from Thabo Mofutsanyana and 178 from Xhariep district. All 320 bovine carcasses from which blood samples were collected for serological analysis were reported free of beef measles by the abattoir meat inspectors (Table 4.9).

Analysis of blood samples with AgELISA gave a total prevalence of 5.6% in three districts. The AgELISA results showed that cattle in Motheo district had a higher 9.5% (7/73) prevalence than those in Thabo Mofutsanyana which had a prevalence of 7% (5/69) and Xhariep with 3% (6/178) (Table 4.9).

Table 4.9: Comparison of MoAb HP10 based AgELISA and meat inspection efficiency in detecting bovine cysticercosis in cattle brought for slaughter at abattoirs in three districts of the Free State Province in South Africa. Abattoirs were classified as high throughput if ≥ 50 animals are slaughtered per day and low throughput where ≤ 49 animals are slaughtered per day; the number of positives (x) found out of those sampled (y) and the percent is indicated in brackets (%) i. e % (x/y).

Districts and abattoirs	**Low/high throughput**	**ELISA results**	**Meat inspection results**
Motheo		**9.5% (7/73)**	**0% (0/73)**
A	Low	0% (0/16)	0% (0/16)
C	High	10% (5/50)	0% (0/50)
B	Low	28% (2/7)	0% (0/7)
Thabo Mofutsanyana		**7% (5/69)**	**0% (0/69)**
D	Low	5% (1/19)	0% (0/19)
E	High	8% (4/50)	0% (0/50)
Xhariep		**3% (6/178)**	**0% (0/178)**
I	High	4% (2/50)	0% (0/50)
K	Low	0% (0/35)	0% (0/35)
L	Low	6% (3/49)	0% (0/49)
J	Low	2% (1/44)	0% (0/44)
Total	**6 Low-; 3 High throughput**	**5.6% (18/320)**	**0% (0/320)**

CHAPTER 5: DISCUSSION, GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

All three extraction methods employed in this study were found to be very efficient in yielding DNA suitable for PCR amplification. During optimisation of conventional PCR assays, the 600 bp HDP2 gene was successfully amplified in the *T. saginata* positive control, but failed to amplify in field samples. This could have possibly been caused by the inhibitors that interfere with PCR amplification. According to Kalle *et al*. (2014) the manner in which the sample is prepared affects the quality and concentration of DNA templates which in turn directly affects the outcome of PCR amplifications. The different stages of the parasite could have effect on the detection of the target 600 bp HDP2 gene, since DNA of the positive control was extracted from the tapeworm while DNA of the field samples was extracted from the metacestode or the cysticercus stage.

Furthermore, the second assay failed to amplify the 170 bp HDP2 gene in DNA extracted from both the control and field samples. This calls for further optimisation experiments for this gene.

HotStar *Taq* PCR assay together with a different My *Taq* PCR assay was also optimised for detection of the 238 bp mitochondrial cytochrome c oxidase subunit I (*cox*1) gene. The assays detected the target gene in the *T. saginata* positive control sample which was extracted by different extraction methods (phenol chloroform, High pure PCR template preparation (Roche Diagnostics, Mannheim, Germany) and QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany) kits. However, the target gene was not observed in the phenol chloroform extraction in HotStar *Taq* PCR assay but was detected in the extractions by High pure PCR template preparation (Roche Diagnostics, Mannheim, Germany) and QIAamp DNA Mini kit (Qiagen Pty Ltd., Hilden, Germany) kits. The target gene was however, amplified in all three extractions in My *Taq* PCR assay, but with a very faint band being observed in the phenol chloroform extraction. The target *cox*1 gene was detected in all different volumes ranging between 0.5 and 5 µl with no amplification of the nonspecific DNA band.

Detection limit of the My *Taq* PCR assay was determined on amplification of both *T. saginata* and *T. solium cox*1 genes. The lower detection limit with *Tsag*-*cox* primer set in the *T. saginata* positive control was 1 pg, whilst the detection limit with *Tsol*-*cox* primer set was 100 pg. These detection limits different from the1 ng detection limit reported by Sreedevi *et al*. (2012) when amplifying the total DNA extracted from the cysticerci of *T. solium* with *cox*1 I and *cox*1 II primer set. This difference could be due to the use of different primers or performing PCR reactions using different thermal cycling programmes or how the sample is prepared (Sreedevi *et al*. 2012).

Some cysts and most of the blood samples showed fragmented DNA bands in them, however the target gene was also detectable in these samples. Appearance of the fragmented DNA could be an indication of the degradation of DNA as suggested by Golenberg *et al*. (1996). Studies suggested that degraded DNA can still be amplified by PCR if the primers are designed to recognize and amplify a shorter target sequence, between 100 and 300 bp, preferably of mitochondrial genes Chiesa *et al*. (2010). Chiesa *et al*. (2010) was able to confirm the diagnosis made at post-mortem inspection in 94.3% (150/159) of degenerated cyst samples and Sreedevi *et al*. (2012) confirmed all nine degenerated cysts being positive for cysticercosis in PCR test.

All the pooled blood samples tested positive for taeniid infections, however when the same samples were tested individually not all of them tested positive. This suggests that the positive samples with high DNA concentration could have masked the negative samples or samples with low DNA in the tested pooled blood samples, as per Rys & Persing (1993) statement.

Even though all 233 porcine blood samples tested negative when screened individually, but the assay has shown the potential of detecting *T. saginata* and *T. solium* infections in blood samples thus potential of detecting *T. solium* and *T. saginata* infections in live animals which can be helpful in detecting infection before slaughter. This study demonstrated that PCR test can be used as a confirmatory tool for identifications made at abattoirs and have the potential to be used as a diagnostic tool in blood samples.

My *Taq* PCR assay for detection of the *cox*1 gene can be successfully used as a confirmatory diagnostic tool as described in Appendix 2. Furthermore, PCR assays for detection of the HDP2 genes needs to be further optimised to ensure optimal conditions for amplification of the 600 bp and 170 bp in the field samples. Since inhibitors have shown to have an effect on the sensitivity of the PCR, precautions such as preparing reagents and working in a sterile environment must be taken into consideration to avoid unsuccessful detection of the target gene.

The real-time PCR developed in the present study was able to detect and discriminate the two *Taenia* species, *T. saginata* and *T. solium*. Both *T. saginata* and *T. solium* qPCR assays were successfully optimised, since their standard curves produced 98% PCR efficiencies which is close to a recommended 100% PCR efficiency for an optimised assay (Ginzinger 2002).

*T*aenia *saginata* tapeworm alone was used for determining the sensitivity of the assay since there was no positive control from the *T. saginata* cysticercus. The *T. saginata* real-time PCR assay showed that it can detect the gene of interest up to the concentration of 0.00013 ng/µl (1.3 pg). While *T. solium* real-time PCR assay indicated can detect the gene of interest up to the concentration of 0.0034 ng/µl (3.4 pg).

The detection limits of the currently developed *T. saginata* and *T. solium* assays differ from the ones recorded in literature. Cuttell *et al*. (2013) reported a detection limit of 1 fg of *T. saginata* DNA corresponding to a Ct value of 35.09 using total DNA extracted from cysticerci with the cytochrome c oxidase subunit 1 (CO1) gene real-time PCR assay. The detection limit of the current study was higher than that reported in Cuttell *et al*. (2013) study. Praet *et al*. (2013) also reported the lowest detection limits of 1 and 2.5 fg of *T. solium* and *T. saginata* respectively when detecting the ribosomal Internal Transcribed Spacer 1 (ITS1) sequences of the two parasites in faecal samples when developing real-time polymerase chain reaction assay (copro-PCR). The variation in the detection limits in the present study could be due to the differences in sample preparation, DNA extraction method or thermal cycling conditions used for amplification of the target gene or detection of a different gene as suggested by Sreedevi *et al*. (2012).

The good specificity of the *T. saginata* primers was shown by not cross-reacting with any genomic DNAs from a panel including the host muscles of beef and pork and other related *Taenia* species such as *T. solium* and *T. hydatigena*. Fluorescence signals produced only from the *T. saginata* positive control DNA was an indication of good specificity of the *T. saginata* primers. The *T. solium* primers were also specific for *T. solium* only as is the tested DNA of *T. saginata*, *T. hydatigena* and host muscles of beef and pork tested negative when *T. solium* primers were used.

The assays respectively confirmed 63% (45/71) and 100% (2/2) *T. saginata* and *T. solium* cysticerci. Failure of the developed *T. saginata* real-time PCR assay to detect all 71 bovine cyst samples could have been caused by cyst degeneration as PCR sensitivity decreases with cyst degeneration (Cuttell *et al*. 2013). Most of the cysts appeared to be calcified with their contents being solid (Abuseir *et al*. 2006). Similarly, the real-time PCR assay developed by Cuttell *et al*. (2013) which targets the 131 bp COI gene of *T. saginata* failed to detect as many positive samples. The assay detected 100% of viable and caseated cysts, but failed to detect 1/16 calcified and 2/3 highly degenerated samples. The concentrations of the extracted DNA samples were sufficient enough to detect the target DNA also the quality of most of the DNA samples were in the $OD_{260/280}$ ratio of 1.8-2.0 indicates that the absorption was due to nucleic acid and the $OD_{260/280}$ ratio of 18 samples were below 1.7 which was an indication of protein contamination. Since the quality and the quantity of DNA also have effect on PCR assay, the negative results could be caused by DNA degradation as a result of freezing and thawing of the samples and also storing of samples for longer period.

This is the first study to report on development of a *T. solium* qPCR assay, hence no comparison can be made with regard to its detection efficiencies.

Both the newly developed assays showed potential in detecting *Taenia* infections in the blood as they detected 75% (458/614) of *T. saginata* and 33% (76/233) of *T. solium* infections in bovine and porcine blood samples. In contrast to amplification curves of both *T. saginata* and *T. solium* positive control samples which fluoresced before cycle 30, the amplification curves of all blood samples fluoresced after cycle 30. This late detection of the target gene in the blood samples could be caused by less DNA concentration as compared to DNA of the positive control sample (Bustin *et al*. 2009). Furthermore, DNA degradation is another possible factor that could have affected the detection performance of the assay.

The results of the study suggest however that the assay can be used with confidence when diagnosing viable and degenerated cysts and are consistent with degree of viability as a factor in PCR sensitivity. The assay was less sensitive but offered advantages of faster turnaround times and reduced contamination risk. Estimates of the assay's repeatability and reproducibility showed the assay is highly reliable with reliability coefficients ranging from 0.974 to 0.988 (Wong & Medrano 2005). Advantages of the new assay when compared with the currently described molecular methods is that is rapid, reproducible diagnosis and has a reduced risk of contamination by using a closed system (Cuttell *et al*. 2013).

The present study has further demonstrated that the newly developed *T. saginata* and *T. solium* qPCR assays can be used as a confirmatory tool for identifications made at abattoirs. Even though the sensitivity of the real-time PCR assay was low when screening the field (blood) samples, it has proved that it has the potential of being a good ante-mortem diagnostic tool that can be used for diagnosis of *Taenia* infections in animals before they can be slaughtered and furthermore preventing economic loss due to cysticercosis. Since the sensitivity of PCR decreases with degeneration of cysts, the correct storage of the cyst samples could help in improving the sensitivity of real-time PCR assay. Furthermore, PCR inhibition can be avoided by analysing blood samples earlier before hemolysis of the red blood cells which has a negative effect on PCR efficiency.

The HP10 AgELISA (Harrison *et al*. 1989) used to detect bovine cysticercosis infection in the blood of the cattle brought to slaughter at the Free State abattoirs revealed an overall bovine cysticercosis sero-prevalence of 5.6% which was high compared to the 0% obtained from meat inspection records. Motheo district showed the highest sero-prevalence of 9.5% (7/73) followed by Thabo Mofutsanyana with 7% (5/69) and lastly Xhariep district with 3% (6/178). Since epidemiological data on bovine cysticercosis is scarce in South Africa it is difficult to compare the level of infection between Provinces. In Southern Africa, surveys on the epidemiology of bovine cysticercosis in cattle have been carried out in Botswana, Zambia and Zimbabwe (Mosienyane 1986; Pugh and Chambers 1989; Giesecke 1997; Dorny *et al*. 2002). Dzoma *et al*. (2011) reported a prevalence of 0.2 in North-West Province, South Africa by meat inspection which is different from the method used in the present study.

The prevalence found in the study clearly indicates that bovine cysticercosis is present in the study area and cattle that were brought to slaughter have the circulating antigen HP10. The results furthermore indicate that carcasses with bovine cysticercosis are being passed on for human consumption since the meat inspectors did not report any positive cases of beef measles in the slaughtered animals during the collection of the blood samples for the study.

Free State is the third-largest Province in South Africa and has a population size of 5.3%. When focusing on each district of the Free State Province, Motheo district has population size of 1.4% with 0.02% household having access to flush toilets connected to the sewerage and 0.03% have access to piped water. Thabo Mofutsanyana with population size of 1.4% with 0.03% having access to flush toilet connected to sewerage and 0.04% with piped water inside their homes. In a population size of 0.3% in Xhariep district, 0.004% have access to flush toilets and 0.007% have access to piped water (www.localgoverment.co.za). The reason for 5.6% sero-prevalence found in the three districts of the Free State Province could be the result of inadequate sanitation since not all households have access to basic sanitation. Contamination by defecation of humans infected with taeniosis in pastures or water which is used by the animals for drinking can lead to the transmission of the infection to animals (Eshitera *et al*. 2012). Most of the animals brought for slaughter were from the commercial farms, since no information was enquired about hygiene and availability of toilets, then it is possible that the animals could have been infected with grazing on pastures contaminated with tapeworm eggs from human faeces.

Wastewater management practices through irrigation of pasture may cause contamination of grazing land with taeniid eggs which are further spread through rainfall by washing large land areas that might also be contaminated with human waste and end up contaminating surface water such as rivers, lakes and dams which are accessible to animals thus resulting in infection of the animals. Therefore, water access of animals to these could have also contributed to the infection of the animals since it was reported as one of the factors responsible for transmission of the disease and not all areas have access to clean water. The study done in Qwaqwa in Thabo Mofutsanyana district on assessment of water quality also proved that the quality of the water in the rivers of that area is poor due to faecal contamination as a result of wastewater with municipality sewer discharge effluent contained in Qwaqwa rivers (Motholo *et al*. 2015).

The 2011 Free State Province census showed that a total of 1 419 households in Motheo, 11 600 in Thabo Mofutsanyana and 1 036 in Xhariep districts are still using the bucket system (www.localgoverment.co.za) which can be also contributing to occurrence of bovine cysticercosis in the Free State Province. Most of the animals brought for slaughter were from the commercial farmers. If by any chance that there is a possibility that the bucket system waste is used to irrigate or fertilize feed crops and pastures therefore chances of animals to become infected are very high (SCVPH 2000).

Population size has been suggested to be a risk factor as higher population density can increase the risk of bovine cysticercosis (Boone *et al*. 2007). The high population density in Motheo district could have contributed to occurrence of high prevalence of bovine cysticercosis in that area. Age of animals that were brought to the abattoirs was not considered in the present study and it also needs to be looked into since the risk of exposure increases with the age of the animal (Dorny & Praet 2007; Dorny 2000). Furthermore, the gender of the animals needs to be considered when determining prevalence since females are more susceptible than males, they are used as replacement breeders and this causes them to stay longer on the farm hence getting more exposure (Dzoma *et al*. 2011).

The study demonstrated that meat inspection is less sensitive for the diagnosis of cysticercosis in infected carcasses as in agreement with the findings of Onyango-Abuje *et al*. (1996a) and the findings reported by Assava *et al*. (2009). The use of antigen detecting ELISA showed that it can be used as ante-mortem diagnostic tool which can detect infected animals before being slaughtered. The test can also be useful in diagnosing cysticercosis in herds to help in locating areas where infections occurred as a result this could assist the farmers to practice control measures such as treatment or reduce field contamination by human faeces.

5.2 GENERAL CONCLUSIONS

Bovine and porcine cysticercosis like other metacestodes in livestock are zoonoses of socioeconomic and public health importance (Asaava *et al*. 2009). Cattle infected with *Taenia saginata* cysticerci and pigs with *T. solium* cysticerci play a role in the transmission and maintenance of human taeniosis (Pinto *et al*. 2000). Taeniosis and cysticercosis remains a public health concern in many developing countries of Latin America, Africa and Asia (Eshitera *et al*. 2012).

This study has successfully evaluated more specific and sensitive assays for detection of bovine and porcine cysticercosis. PCR detecting the 600 bp HDP2 gene was successful in the positive control sample only but was not optimised to a level that it can detect the target gene in the field samples. The same challenge was observed when detecting the 170 bp HDP2 gene in both positive and field samples. The successful detection of these genes occurred when they were detected simultaneously in a multiplex PCR established by Gonzalez *et al*. (2002). However, the detection of these genes was not successful when individually detection was tried in separate PCR assays. More investigations on optimisation still need to be focused on determination of conditions which function efficiently for detection of the HDP2 genes in the field samples.

The optimized conventional and developed real-time PCR assays in this study proved to be specific and sensitive molecular tools for confirmation of post-mortem diagnosis of *T. solium* and *T. saginata* cysticercosis and in identifying suspected cysts in slaughtered cattle and pigs. The *Taq*Man probe real-time PCR assays of the two parasites have shown a potential that they can be used in rapid and routine detection of *T. solium* and *T. saginata* cysticercosis.

The sensitivities of the assays were not similar to those reported by other authors due to different preparations of the samples and different procedures followed when performing the PCR reactions (Sreedevi *et al*. 2012). Conventional PCR and real-time PCR assays for *T. saginata* and *T. solium* used in this study showed similar detection limit when detecting the target *cox*1 gene at the same DNA concentrations. However, the detection limit of *T. solium* in the conventional PCR assay was low when compared to the sensitivities of real-time PCR assays for *T. saginata* and *T. solium*. The assays showed good specificity by not cross-reacting with any genomic DNAs from the host and other organisms commonly misidentified *as T. saginata* or *T. solium* cysticerci. Furthermore, the *cox*1 gene of *T. saginata* or *T. solium* was able to be detected in DNA extracted from blood samples.

Different sensitivities were observed when the two assays were used to screen the field samples. Because the PCR sensitivity depends on degree of viability of the cyst samples, since PCR sensitivity decreases with cyst degeneration (Cuttell *et al*. 2013), then the negative results obtained during the validation of the assays when screening the field cyst samples could be due to DNA degradation. Furthermore, failure of the conventional PCR assay to amplify the target gene in the porcine blood samples could be caused by the presence of the PCR inhibitors in the DNA which are known to interfere with DNA amplification by affecting or binding *Taq* or by binding DNA template (McCord *et al*. 2011). Inhibition can be avoided by dilution of the template DNA, further purification by agarose gel electrophoresis or various columns and resins intended for DNA purification, or addition of substances like bovine serum albumin to the PCR reaction mixture. PCR inhibition can be identified by addition of an internal positive control to each PCR reaction. However, the use of DNA dilutions to dilute out the PCR inhibitors also dilutes the DNA present and in cases where the target organism is present in low numbers, this practice may also produce a false-negative result. Therefore, the addition of an internal positive control to each PCR reaction could help in identifying PCR inhibition (Rintala 2003).

The identification of the suspect cysts, lesions, or cyst residues in domestic animals at the slaughterhouse with molecular assays such as conventional and real-time PCR would aid in the appropriate treatment of the carcasses and in the control of these parasites in domestic livestock. The species specific primers will make these molecular assays to be useful in the differential diagnosis, molecular characterization, and epidemiological surveys of *T. saginata,* and *T. solium.* To date, there is no published real-time PCR assay for diagnosis of *T. solium* in blood and cyst samples and *T. saginata* in blood samples and this is the first study to describe real-time PCR assay for detection of *Taenia* infection in blood.

The results obtained in this study indicate that the monoclonal antibody based ELISA test can be applied for diagnosis of cysticercosis, especially in screening situations. The present study revealed the presence of bovine cysticercosis in some cattle in the Free State Province. The level of cysticercosis obtained with antigen detecting ELISA showed the usefulness of the method for diagnosis of *Taenia* infection in blood of animals and evaluate the identification of meat inspection procedure for detecting cysticercosis in carcasses. The prevalence in this study has demonstrated the occurrence of bovine cysticercosis in the three districts of the Free State Province. Motheo district appeared to have high levels of *Taenia* infections. It is known that in areas where cysticercosis is present people are at risk of acquiring taeniosis and human cysticercosis including the fatal neurocysticercosis. Therefore, both veterinary and medical public health services should determine the main point of the disease transmission and formulate control strategies to reduce the disease risk factors.

5.3 RECOMMENDATIONS

Further PCR optimisation needs to be conducted for the detection of the *T. saginata* and *T. solium* HDP2 genes in the field samples in order to determine which conditions are optimal for amplification of the 600 bp and 170 bp.

The sensitivity of conventional PCR assay in blood samples needs to be improved by analysing them immediately after sample collection so as to avoid hemolysis of the red blood cells that would results in PCR inhibition.

DNA sequencing of PCR amplicons is needed to identify genetic variants such as polymorphisms, rearrangements, translocations in the genes of the two parasites.

Proper storage of cyst and DNA samples needs to be investigated to prevent degradation as this affects detection sensitivity of DNA assays as observed in the current study with detection performance of real-time PCR.

Further experiments need to be conducted to verify the use of newly developed real-time PCR assays for detection *T. saginata* and *T. solium* from blood.

Based on the results obtained in the monoclonal (McAb) antibody antigen detecting ELISA, further epidemiological studies in the rural communities where the animals are raised should also be assessed for *Taenia* infections.

Educational programmes should be initiated to build awareness on the transmission *Taenia* parasites and dynamics of cysticercosis, as well as creation of strong primary health care network that would help in maintaining hygienic and sanitary conditions in the communities.

REFERENCES

ABUSEIR, S., KÜHNE, M., T. SCHNIEDER, T., KLEIN, G. & EPE, C. 2007. Evaluation of a serological method for the detection of *Taenia saginata* cysticercosis using serum and meat juice samples. *Parasitology Research*, **101:**131-137.

AL-SOUD, W.A. & RÅDSTROM, P. 1998. Capacity of nine thermostable DNA Polymerases to mediate DNA Amplification in the Presence of PCR-Inhibiting Samples. *Applied and Environmental Microbiology*, **64:** 3748-3753.

ASAAVA, L.L., KITALA, P.M., GATHURA, P.B., NANYINGI, M.O., MUCHEMI, G. & SCHELLING, E. 2009. A survey of bovine cysticercosis/ human taeniosis in Northern Turkana District, Kenya. *Preventative Veterinary Medicine*, **89:** 197-204.

ASSANA, E., KYNGDON, C.T., GAUCI, C.G., GEERTS, S., DORNY, P., DE DEKEN, R., ANDERSON, G.A., ZOLI, A.P. & LIGHTOWLERS, M.W. 2010. Elimination of *Taenia solium* transmission to pigs in a field trial of the TSOL18 vaccine in Cameroon. *International Journal for Parasitology*, **40:** 515-519.

BOONE, I., THYS, E., MARCOTTY, T., BORCHGRAVE, J., DUCHEYNE, E. & DORNY, P. 2007. Distribution and risk factors of bovine cysticercosis in Belgian dairy and mixed herds. *Preventative Veterinary Medicine*, **82:** 1-11.

BRANDT, J. R. A., GEERTS, S., DE DEKEN, R., KUMAR, V., CEULEMANS, F., BRIJS, L. & FALLA, N. 1992. A monoclonal antibody-based ELISA for the detection of circulating excretory-secretory antigens in *Taenia saginata* cysticercosis. *International Journal of Parasitology*, **22:** 47-477.

BRAAE, U.C., KABULULU, M., NØRMARK, M.E., NEJSUM, P., NGOWI, H.A. & JOHANSEN, M.V. 2015. *Taenia hydatigena* cysticercosis in slaughtered pigs, goats, and sheep in *Tanzania. Tropical Animal Health and Production*, **47:** 1523-30.

BUSTIN, S.A., BENES, V., GARSON, J.A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M.W., SHIPLEY, G.L., VANDESOMPELE, J. & WITTWER, C.T. 2009. The MIQE Guidelines: Minimum information for publication of *q*uantitative real-time PCR experiments. *Clinical Chemistry*, **55:** 611-22.

CABARET, J., GEERTS, S., MADELINE, M., BALLANDONNE, C. & BARBIER. D. 2002. The use of urban sewage sludge on pastures: the cysticercosis threat. *Veterinary Research*, **33:** 575-597.

CAI, X., YUAN, G., ZHENG, Y., LUO, X., ZHANG, S., DING, J., JING, Z. & LU C. 2008. Effective production and purification of the glycosylated tsol18 antigen, which is protective against pig cysticercosis. *Infection and Immunity*, **76:** 767-770.

CEDERBERG, S., SIKASUNGE, C.S., ANDERSSON, A. & JOHANSEN, M. V. 2012. Short Communication: In vitro efficacy testing of Praziquantel, Ivermectin, and Oxfendazole against *Taenia Solium* cysts. *Journal of Parasitology Research*, 2012:363276. doi**:** 10.1155/2012/363276.

CHAPMAN, A., VALLEJO, V., MOSSIE, K. G., ORTIZ, D., AGABIAN, N. & FLISSER, A. 1995. Isolation and characterization of species-specific DNA probes from *Taenia solium* and *Taenia saginata* and their use in an egg detection assay. *Journal of Clinical Microbiology*, **33:** 1283-1288.

CHIESA, F., DALMASSO, A., BELLIO, A., MARTINETTI, M., GILI, S. & CIVERA, T. 2010. Development of a biomolecular assay for post-mortem diagnosis of *Taenia saginata* cysticercosis. *Foodborne Pathogens and Disease*, **7:** 1171-1175.

CUTTELL, L., OWEN, H., LEW-TABOR, A.E. & TRAUB, R.J. 2013. Bovine cysticercosis--development of a real-time PCR to enhance classification of suspect cysts identified at meat inspection. *Veterinary Parasitology*, **194:** 65-69.

DA SILVA, M.R.M., UYHARA, C.N.S., SILVA, F.H., ESPINDOLA, N.M., POLETI, M.D., VAZ, A.J., MEIRELLES, F.V. & MAIA, A.A.M. 2012. Cysticercosis in experimentally and naturally infected pigs: Parasitological and immunological diagnosis. *Pesquisa Veterinária Brasileira*, **32:** 297-302.

DA SILVA, A.J., ZANGIROLAM, T.C., NOVO-MANSUR, M.T.M., GIORDANO, R.C. & MARTINS, E.A.L. 2014. Live bacterial vaccine vectors: An overview. *Brazilian Journal of Microbiology*, **45:** 1117-1129.

DESJARDINS, P. & CONKLIN, D. 2010. NanoDrop Micro-volume Quantitation of Nucleic Acids. *Journal of Visualized Experiments*, pii: 2565. doi: 10.3791/2565.

DIAS, A.K.K., AOKI, S. M., GARCIA, J.F. & NUNES, C.M. 2007. *Taenia solium* and *Taenia saginata*: Identification of sequence characterized amplified region (SCAR) markers. *Experimental Parasitology*, **117:** 9-12.

DORNY, P., VERCAMMEN, F., BRANDT, J., VANSTEENKISTE, W., BERKVENS, D. & GEERTS, S. 2000. Sero-epidemiological study of *Taenia saginata* cysticercosis in Belgian cattle. *Veterinary Parasitology,* **88:** 43-49.

DORNY, P., PHIRI, I, GABRIEL, S., SPEYBROECK, N. & VERCUYSSE, J. 2002. A sero-epidemiological study of *Taenia saginata* cysticercosis in Zambia cattle. *Veterinary Parasitology*, **104:** 211-215.

DORNY, P., BRANDT, J., ZOLI A. & GEERTS, S. 2003. Immunodiagnostic tools for human and porcine cysticercosis. *Acta Tropica*, **87:** 79-86.

DORNY, P. & PRAET, N. 2007. *Taenia saginata* in Europe. *Veterinary Parasitology*, **149:** 22-24.

DUTRA, L.H., GIRROTO, A., DA COSTA VIEIRA, R.F., VIERA, T.S.W.J., ZANGIROLAMO, A.F., MARQUES, F.A.C., HEADLEY, S.A. & VIDOTTO O. 2012. The prevalence and spatial epidemiology of cysticercosis in slaughtered cattle from Brazil. *Semina: Ciências Agrárias, Londrina*, **33:** 1887-1896.

DZOMA, B.M., SETLHODI, E.K., MOLEFE, M.M., MOTSEI, L.E., BAKUNZI, F.R., NDOU, R.V. & NYIRENDA, M. 2011. Prevalence of bovine cysticercosis in the North West Province of South Africa from 2000 to 2010. *Journal of Human Ecology*, **36:** 9-12.

EICHENBERGER, R.M., LEWIS, F., GABRIËL, S., DORNY, P., TORGERSON, P.R. & DEPLAZES, P. 2013. Multi-test analysis and model-based estimation of the prevalence of *Taenia saginata* cysticercus infection in naturally infected dairy cows in the absence of a 'gold standard' reference test. *International Journal for Parasitology*, **43:** 853-859.

ESHITERA, E.E., GITHIGIA, S.M., KITALA, P., THOMAS, L.F., FÈVRE, E.M., HARRISON, L.J.S., MWIHIA, E.W., OTIENO, R.O., OJIAMBO, F. & MAINGI, N. 2012. Prevalence of porcine cysticercosis and associated risk factors in Homa Bay District, Kenya. BMC *Veterinary Research*, **8:** 234.

FERRER, E., BENITEZ, L., FOSTER-CUEVAS, M., BRYCE, D., WAMAE, L.W., ONYANGO-ABUJE, J.A., GARATE, T., HARRISON, L.J.S. & PARKHOUSE, R.M.E. 2003. *Taenia saginata* derived synthetic peptides with potential for the diagnosis of bovine cysticercosis. *Veterinary Parasitology*, **111:** 83-94.

FERRER, E., M., CORTÉZ, M.M., CABRERA, Z., ROJAS, G., DÁVILA, I., DE NOYA, B.A., PÉREZ, H.A., FERNANDEZE, I., ROMERO, H.U., LESLIE J.S. HARRISON, L. J.S., R. MICHAEL E. PARKHOUSE, R.M.E. & GÁRATEI, T. 2005. Oncospheral peptide-based ELISAs as potential sero-epidemiological tools for *Taenia solium* cysticercosis/neurocysticercosis in Venezuela. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **99:** 568-576.

FERRER, E., GONZÁLEZ, L.M., MARTÍNEZ-ESCRIBANO, J.A., GONZÁLEZ-BARDERAS, M.E., CORTÉZ, M.M., DÁVILA, I., HARRISON, L.J.S., PARKHOUSE, R.M.E. & GÁRATE, T. 2007. Evaluation of recombinant HP6-Tsag, an 18 kDa *Taenia saginata* oncospheral adhesion protein, for the diagnosis of cysticercosis. *Parasitology Research*, **101:** 517-25.

FLISSER, A. & LIGHTOWLERS, M.W. 2001. Vaccination against *Taenia solium* cysticercosis. *Memorias do Instituto Oswaldo Cruz*, **96:** 353-6.

FLISSER, A., GAUCI, C.G., ZOL, E A., MARTINEZ-OCANA, J., GARZA-RODRIGUEZ, A., DOMINGUEZ-ALPIZAR, J., MARAVILLA, P., RODRIGUEZ-CANUL, R., GUILLERMINA, A., AGUILAR-VEGA, L., KYNGDON, C., GEERTS, S. & LIGHTOWLERS, M. 2004. Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infection and. Immunity*, **72:** 5292-5297.

GARCÍA, H.H., GONZALEZ, A.E., EVANS, C.A., GILMAN, R.H. & CYSTICERCOSIS WORKING GROUP IN PERU. 2003. *Taenia solium* cysticercosis, *Lancet*, **362:** 547-56.

GAREDAGHI, Y., SABER, R. & KHOSROSHAHI, M.S. 2011. Prevalence of bovine cysticercosis of slaughtered cattle in Meshkinshahr abattoir. *American Journal of Animal and Veterinary Sciences*, **6:** 121-124.

GAUCI, C.G., JAYASHI, C.M., GONZALEZ, A.E., LACKENBY, J. & LIGHTOWLERS, M.W. 2012. Protection of pigs against *Taenia solium* cysticercosis by immunization with novel recombinant antigens. *Vaccine*, **540:** 3824-3828.

GEYSEN, D., KANOBANA, K., VICTOR, B., RODRIGUEZ-HIDALGO, R., DE BORCHGRAVE, J., BRANDT, J. & DORNY, P. 2007. Validation of meat inspection results for *Taenia saginata* cysticercosis by PCR-restriction fragment length polymorphism. *Journal Food Protection*, **70:** 236-40.

GIESIECKE, W.H. 1997. Prevalence and economic implication of taeniosis or cysticercosis in South Africa. Report of workshop held at the Onderstepoort Veterinary Institute, Onderstepoort, South Africa, 18-19 August, 1997, pp**:** 19-70.

GINZINGER, D.G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology*, **30:** 503-512.

GOLENBERG, E., M., BICKEL, A. & WEIHS P. 1996. Effect of highly fragmented DNA on PCR. *Nucleic Acids Research*, **24:** 5026-5033.

GONZA´LEZ, A.E., VERASTEGUI, M., NOH, J.C., GAVIDIA, C., FALCON, N., BERNAL, T., GARCIA, H.H., TSANG, V.C., GILMAN, R.H. & WILKINS, P.P. 1999. Persistence of passively transferred antibodies in porcine *Taenia solium* cysticercosis. Cysticercosis Working Group in Peru. *Veterinary Parasitology*, **86:** 113-118.

GONZA´LEZ, L.M., MONTERO, E, HARRISON, L.J.S., PARKHOUSE, R.M.E. & GARATE, T. 2000. Differential Diagnosis of *Taenia saginata* and *Taenia solium* Infection by PCR. *Journal of Clinical Microbiology*, **38:** 737-744.

GONZÁLEZ, L.M., MONTERO, E., PUENTE, S., LÓPEZ-VELEZ, R., HERNÁNDEZ, M., SCIUTTO, E., HARRISON, L.J., PARKHOUSE, R.M. & GÁRATE, T. 2002. PCR tools for the differential diagnosis of *Taenia saginata* and *Taenia solium* taeniosis/cysticercosis from different geographical locations. *Diagnostic Microbiology and Infectious Disease*, **42:** 243-9.

GONZÁLEZ, L.M., VILLALOBOS, N, MONTERO, E., MORALES, J., SANZ, R.A., MURO, A., HARRISON, L.J., PARKHOUSE, R.M. & GÁRATE, T. 2006. Differential molecular identification of Taeniid spp. and Sarcocystis spp. cysts isolated from infected pigs and cattle. *Veterinary Parasitology*, **142:** 95-101.

GONZÁLEZ, L.M., BAILO, B., FERRER, E., GARCIA, M. D. F., HARRISON, L. J. S., PARKHOUSE, M.R.E. & GÁRATE, T. 2010. Characterization of the *Taenia* spp HDP2 sequence and development of a novel PCR-based assay for discrimination of *Taenia saginata* from *Taenia asiatica*. *Parasites & Vectors*, **3:** 51.

GREENE, R.M., WILKINS, P.P. & TSANG, V.C.W. 1999. Diagnostic glycoproteins of *Taenia solium* cysts share homologous 14 and 18 KDa subunits. *Molecular and Biochemical Parasitology*, **99:** 257-261.

GRUNENWALD, H. 2003. Optimisation of polymerase chain reaction. *Methods in Molecular Biology*, **226:** 89-99.

GUO, A., JIN, Z., ZHENG, Y., HAI, G., YUAN, G., LI, H. & CAI, X. 2007. Induction of protection against porcine cysticercosis in growing pigs by DNA vaccination. *Vaccine*, **25:** 170-175.

HARRIS, S. & JONES, D.B.1997. Optimisation of the Polymerase Chain Reaction. *British Journal of Biomedical Science*, **54:** 166-173.

HARRISON, L.J., JOSHUA, G.W., WRIGHT, S.H. & PARKHOUSE, R.M.E. 1989. *Parasite Immunology*, **11:** 351-70.

HARRISON, L. J. S., DELGADO, J. & PARKHOUSE, R. M. E. 1990. Differential diagnosis of *Taenia saginata* and *Taenia solium* with DNA probes. *Parasitology*, **100:** 459-461.

HARRISON, G,B., HEATH, D,D., DEMPSTER, R.P., GAUCI, C, NEWTON., S.E., CAMERON, W.G., ROBINSON, C.M., LAWRENCE, S.B., LIGHTOWLERS. M., W. & RICKARD, M., D. 1996. Identification and cDNA cloning of two novel low molecular weight host-protective antigens from *Taenia ovis* oncospheres. *International Journal for Parasitology*, **26:** 195-204.

http://www.vacunasyviajes.es/vacunasyviajes/Teniasis_Atlas.html. Taeniosis (accessed on the 24 May 2014).

http://atlas.or.kr/atlas/alphabet_viewp?my_codeName=Taenia%20solium. Web Atlas of medical parasitology (accessed on the 27 May 2014).

http://aoin.eu/wiki/index.php?title=Orange_Free_State. Orange Free State (accessed on the 05 April 2014).

http://www.localgoverment.co.za. The local government handbook: Municipalities of South Africa (accessed on the 12 January 2016).

ITO, A., WANDRA, T., YAMASAKI, H., NAKAO, M., SAKO, Y., NAKAYA, K., MARGONO, S.S., SUROSO, T., GAUCI, C. & LIGHTOWLERS, M.W. 2004. Cysticercosis/taeniosis in Asia and the Pacific. *Vector-borne and Zoonotic Diseases*, **4:** 95-107.

KALLE, E., KUBISTA, M. & RENSING, C. 2014. Multi-template polymerase chain reaction. Biomolecular Detection and Quantification **2:** 11-29.

KANDIL, O.M., NASR, S.M., MAHMOUD, M.S., NASSAR, S.A., EL-METANAWEY, T.M., ABD EL-AZIZ M.H.M. & ABU EL-EZZ, N.M.T. 2012. Serological and biochemical studies on cattle naturally infested with *Taenia saginata* cysticercosis. *Global Veterinaria*, **9:** 571-579.

KARA, M. & DOGANAY, A. 2005. Investigation of antigenic specificity against *Cysticercus tenuicollis* cyst fluid antigen in dogs experimentally infected with *Taenia hydatigena*. *Turkish Journal of Veterinary and Animal Sciences*, **29:** 835-840.

KARSHIMA, N. S., PAM, V. A., BOBBO, A. A. & OBALISA, A. 2013. Occurrence of Cysticercus bovis in cattle slaughtered at the Ibi slaughter house, Ibi Local Government Area of Taraba State, Nigeria. *Journal of Veterinary Advances*, **3:** 130-134.

KEBEDE, N. 2008. Cysticercosis of slaughtered cattle in north-western Ethiopia. *Research in Veterinary Science*, **85:** 522-6.

KUMAR, A. & TADESSE, G. 2011. Bovine cysticercosis in Ethiopia: a review. *Ethiopian Veterinary Journal*, **15:** 15-35.

LIGHTOWLERS, M.W., ROLFE, R. & GAUCI, C.G. 1996. *Taenia saginata* vaccination against cysticercosis in cattle with recombinant oncosphere antigens. *Experimental Parasitology*, **84:** 330-338.

LIGHTOWLERS, M.W. 2010. Eradication of *Taenia solium* cysticercosis: a role for vaccination of pigs. *International Journal of Parasitology*, **40:** 1183-92.

MCCORD, B., OPEL, K., FUNES, M., ZOPPIS, S. & JANTZ L.M. 2011. An Investigation of the Effect of DNA Degradation and Inhibition on PCR Amplification of Single Source and Mixed Forensic Samples. https://www.ncjrs.gov/pdffiles1/nij/grants/236692.pdf. (Accessed on the 03 Novenber 2015).

MCINTOSH, A. & MILLER, D. 1960. Bovine cysticercosis with special reference to the early development stages of *Taenia saginata*. *American Journal of Veterinary Research*, **21:** 169-177.

MEGERSA, B., TESFAYE, E., REGASSA, A., ABEBE, R. & ABUNNA, F. 2010. Bovine cysticercosis in cattle slaughtered at Jimma municipal abattoir, south western Ethiopia: Prevalence, cyst viability and its socio-economic importance. *Veterinary World*, **3:** 257-262.

MEIRY, M., BRENNER, G., MARKOVITCS, A. & KLEMENT, E. 2013. A change in the epidemiology of bovine cysticercosis in Israel between 1973 and 2008 due to import of live cattle. *Transboundary and Emerging Disease*, **60:** 298-302.

MINOZZO, J.C., GUSSO, R.L.F., CASTRO, E.A., LAGO, O. & SOCCOL, V.T. 2002. Experimental bovine infection with *Taenia saginata* eggs: ecovery rates and cysticerci location. *Brazilian Archives of Biology and Technology*, **45:** 451-455.

MORALES, J., MARTI´NEZ, J.J., MANOUTCHARIAN, K., HERNA´NDEZ, M., FLEURY, A., GEVORKIAN, G., ACERO, G., BLANCAS, A., TOLEDO, A., CERVANTES, J., MAZA, V., QUET, F., BONNABAU, H., DE ALUJA, A.S., FRAGOSOB, G., LARRALDE, C & SCIUTTO, E. 2008. Inexpensive anti-cysticercosis vaccine: S3Pvac expressed in heat inactivated M13 filamentous phage proves effective against naturally acquired *Taenia solium* porcine cysticercosis. *Vaccine*, **26:** 2899-2905.

MOSIENYANE, M.G. 1986. A survey of *Cysticercus bovis* (measles) infestation in cattle sent for slaughter to Botswana Meat Commission (BMC); a ten years retrospective study-1974-1983. *International Journal of Zoonoses*, **13:** 124-130.

MOTHOLO, L. M., TSOTETSI A. M., NKHEBENYANE, J. S., MOKOATSI, T. E. & THEKISOE, O. M. M. 2015. Assessment of water quality and documentation of macro- and mico- inverterbrates in dams and rivers of Qwaqwa, South Africa. *Report of symposium held at the 7th International Toxicology Symposium in Africa,* Johannesburg, South Africa, 31 August, 2015, pp**:** 22-23.

MURRELL, K.D. 2005. Epidemiology of taeniosis and cysticercosis. In: Murrell, K.D. (Ed.), WHO/FAO/OIE Guidelines for the Surveillance, Prevention and Control of Taeniosis/Cysticercosis. World Health Organisation for Animal Health (OIE), Paris, pp. 27-43.

NAVARRO, E., SERRANO-HERAS, G., CASTAÑO, M.J. & SOLERA, J. 2015. Real-time PCR detection chemistry. *Clinica Chimica Acta*, **439:** 231-250.

NKOUAWA, A., SAKO, Y., NAKAO, M., NAKAYA, K. & ITO, A. 2009. Loop-Mediated Isothermal Amplification Method for Differentiation and Rapid Detection of *Taenia* Species. *Journal of Clinical Microbiology*, **47:** 168-174.

NOURANI, H., PIRALI KHEIRABADI, K.H., RAJABI, H. & BANITALEBI, A. 2010. An unusual migration of *Taenia hydatigena* larvae in a lamb. *Tropical Biomedicine,* **27:** 651-656.

OIE, WORLD ORGANIZATION FOR ANIMAL HEALTH. OIE *Terrestrial Manual* 2014. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2014/pdf/2.09.05_CYSTICERCOSIS (accessed on the 12 March 2015).

OGUNREMI, O. & BENJAMIN, J. 2010. Development and field evaluation of a new serological test for *Taenia saginata* cysticercosis. *Veterinary Parasitology*, **169:** 93-101.

ONYANGO-ABUJE, J.A., HUGHES, G., OPICHA, M., NGINYI, M.K., RUGUTT, K.M., WRIGHT, H.S. & HARRISON, L.J. 1996a. Diagnosis of *Taenia Saginata* cysticercosis in Kenyan cattle by antibody and antigen ELISA. *Veterinary Parasitology*, **61:** 221-230.

ONYANGO-ABUJE, J.A., NGINYI, J.M., RUGUTT, M.K., WRIGHT, S.H., LUMUMBA, P., HUGHES, G. & HARRISON, L.J. 1996b. Sero-epidemiological survey of *T. saginata* cysticercosis in Kenya. *Veterinary. Parasitology*, **64:** 177-185.

PINTO, P.S., VAZ, A.J., GERMANO, P.M. & NAKAMURA, P.M. 2000. Performance of the ELISA test for swine cysticercosis using antigens of *Taenia solium* and *Taenia crassiceps* cysticerci. *Veterinary Parasitology*, **88:** 127-30.

PONDJA, A., NEVES, L., MLANGW, J., AFONSO, S., FAFETINE, J., WILLINGHAM, A.L., THAMSBORG, S.M. & JOHANSEN, M.V. 2010. Prevalence and risk factors of porcine cysticercosis in Ango´ nia District, Mozambique. *PLoS Neglected Tropical Disease*, 4**:** e594.

PRAET, N. 2013. Bayesian modelling to estimate the test characteristics of cropology, copro-antigen ELISA and a novel real-time PCR for the diagnosis of taeniosis. *Tropical Medicine and International Health*, **18:** 608-614.

PUGH, K.E. & CHAMBERS, P.G. 1989. Observations on *Cysticercus bovis* in slaughter cattle in the Matabeleland of Zimbabwe. *Veterinary Record*, **125:** 480-484.

QADEER, M., A. 2008. Prevalence of bovine cysticercosis in jos abattoir, Nigeria. *Animal Research International***, 5: 777-779.**

RAJSHEKHAR, V., JOSHI, D.D., DOANH, N.Q., DE, N.V. & ZHOU, X.N. 2003. *Taenia solium* taeniosis/cysticercosis in Asia: epidemiology, impact and issues. *Acta Tropica*, **87:** 53-60.

RAMAHEFARISOA, R.M., RAKOTONDRAZAKA, M., JAMBOU, R. & CAROD, J.F. 2010. Comparison of ELISA and PCR assays for the diagnosis of porcine cysticercosis. *Veterinary Parasitology*, **173:** 336-9.

RHOADS, M.L, ZARLENGA, D.S. & AL-YAMAN, F.M. 1991. A recombinant immunodiagnostic antigen for bovine cysticercosis. *Southeast Asian ournal of Tropical Medicine and Public Health,* **22:** 268-270.

RINTALA, H. 2003. Streptomycetes in indoor environments - PCR based detection and diversity. Ph.D thesis, University of Kuopio, Kuopio, Finland.

RYS, P., N. & PERSING, D., H. 1993. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. *Journal of ClinicaL Microbiology*, **31:** 2356-2360.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS T. 1989. *Molecular Cloning: A Laboratory Manual, 2nd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SATO, M.O., YAMASAKI, H., SAKO, Y., NAKAO, M., NAKAYA, K., PLANCARTE, A., KASSUKU, A.A., DORNY. P., GEERTS. S., BENITEZ-ORTIZ, W., HASHIGUCH, I Y. & ITO, A. 2003. Evaluation of tongue inspection and serology for diagnosis of *Taenia solium* cysticercosis in swine: usefulness of ELISA using purified glycoproteins and recombinant antigen *Veterinary Parasitology*, **111:** 309-22.

SCANDRETT, W.B. 2007. Improved post-mortem diagnosis of *Taenia saginata* cysticercosis. MSc thesis, University of Saskatchewan, Saskatoon, Saskatchewan.

SCIUTTO, E., MARTINEZ, J.J., VILLALOBOS, N.M., HERNANDEZ, M., JOSE, M.V., BELTRAN, C., RODARTE, F., FLORES, I., BOBADILLA, J.R., FRAGOSO, G., PARKHOUSE, M.E., HARRISON, L.J.S. & DEALUJA, A.S. 1998. Limitations of current diagnostic procedures for the diagnosis of *Taenia solium* cysticercosis in rural pigs. *Veterinary Parasitology*, **79:** 299-313.

SCVPH (Scientific Committee on Veterinary Measures Relating to Public Health). 2000. Opinion on the control of taeniosis/ cysticercosis in man and animals, http://europa.eu.int/comm/food/fs/sc/scv/out36_en.pdf (accessed on the 24 October 2014).

SHORE, G.L. 2001. Diagnostic medical parasitology, 4nd ed. ASM PRESS, Washington, DC, USA.

SIKASUNGE, C.S., PHIRI, I.K., PHIRI, A.M., SIZIYA, S., DORNY, P. & WILLINGHAM, A.L. 2008. Prevalence of *Taenia solium* porcine cysticercosis in the Eastern, Southern and Western Provinces of Zambia. *Veterinary Journal*, **176:** 240-244.

SMITH, H.J., SNOWDON, K.E. & FINLAY, R.C. 1991. Serological diagnosis of cysticercosis by an enzyme-linked immunosorbent assay in experimentally infected cattle. *Canadian Journal of Veterinary Research*, **55:** 274-276.

SREEDEVI, C., HAFEEZ, M., KUMAR, P.A., RAYULU, V.C., SUBRAMANYAM, K.V. & SUDHAKAR, K. 2012. PCR test for detecting *Taenia solium* cysticercosis in pig carcasses. *Tropical Animal Health and Production*, **44:** 95-9.

VAN KERCKHOVEN, VANSTEENKISTE W, CLAES M, GEERTS S, BRANDT J. 1998. Improved detection of circulating antigen in cattle infected with *Taenia saginata* metacestodes. *Veterinary Parasitology*, **76:** 269-74.

WANZALA, W., ONYANGO-ABUJE, J.A., KANG'ETHE, E.K., OCHANDA, H. & HARRISON, L.J. 2002. Sero-diagnosis of bovine cysticercosis by detecting live *Taenia saginata* cysts using a monoclonal antibody-based antigen-ELISA. *Journal of South African Veterinary Association*, **73:** 201-206.

WANZALA, W., ONYANGO-ABUJE, J.A., KANG'ETHE, E.K., ZESSIN, K.H., KYULE, N.M., BAUMANN, M.P.O., OCHANDA, H. & HARRISON, L.J.S. 2003. Control of *Taenia saginata* by post-mortem examination of carcasses. *African Health Sciences*, **3:** 68-76.

WILLIAMS, F.J. 1989. Optimization Strategies for the Polymerase Chain Reaction. *BioTechniques*, **7:** 762.

WONDIMAGEGNEI, K. & BELETE, S. 2015. Prevalence and public health significance of cysticercus bovis in and around Debreberhan City. *European Journal of Applied Sciences*, **7:** 199-208.

WONG, M.L. & MEDRANO, J. F. 2005. Real-time PCR for mRNA quantitation. *BioTechniques*, **39:** 75-85.

YAMASAKI, H., ALLAN, J.C., SATO, M.O., NAKAO, M., SAKO, Y., NAKAYA, K., QIU, D., MAMUTI W., CRAIG, P.S. & ITO, A. 2004. DNA differential diagnosis of Taeniosis and cysticercosis by multiplex PCR. *Journal of Clinical Microbiology*, **42:** 548-553.

ZIRINTUNDA, G. & EKOU, J. 2015. Occurrence of porcine cysticercosis in free-ranging pigs delivered to slaughter points in Arapai, Soroti district, Uganda. *The Onderstepoort Journal of Veterinary Research*, **82:** 888. doi: 10.4102/ojvr.v82i1.888.

APPENDICES

APPENDIX 1

DNA EXTRACTION METHODS

Isolation of genomic DNA from cyst and blood sample using:

Appendix 1A: Phenol chloroform extraction method (Sambrook *et al*. 1989)

Materials

1. 10 mM Tris-HCl [pH 8.0]
2. 10 mM EDTA (Sigma)
3. Proteinase K (Sigma)
4. Phenol chloroform (Sigma)
5. Chloroform (Sigma)
6. Sodium acetate (3 M NaOAc) (Sigma)
7. Isopropanol (Sigma)
8. 70% Ethanol (Mreck)
9. TE buffer (Sigma)
10. 1.5 ml Eppendorf tubes (WhiteHead Scientific)
11. Filtered 1000 µl, 100 µl and 10 µl pipettes (WhiteHead Scientific)
12. Heating block
13. Vortex
14. Centrifuge

Method

1. Lysis buffer preparation: 83ml of double distilled water, 10ml of 10% of SDS stock making a final concentration 1%, addition of 2ml of 0.5M EDTA at pH 8.0 making final concentration 10mM followed by the addition of 1ml of 1M Tris-HCl at pH 8.0 with final concentration of 10mM and the addition of 4ml of 5M NaCl stock of final concentration of 0.2M.
2. Place small pieces of cysts or 50 µl of blood sample in 500 µl of lysis buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate.
3. Add 10 µl of the commercial Proteinase K.
4. Lyse sample overnight in the heating block at +55°C.
5. Add 550 µl of phenol chloroform and mixing and centrifuging at 15 000 rpm for 5 minutes.
6. Add 500 µl of Chloroform to the upper aqueous phase and mixed and centrifuged at 15 000 rpm for 5 minutes.
7. Add 50 µl of Sodium acetate (3 M NaOAc) and 500 µl of Isopropanol to the upper aqueous phase and mixed and centrifuged at 15 000 rpm for 30 minutes.
8. Washed the pellet with 70% Ethanol twice and mixed and centrifuged at 15 000 rpm for 5 minutes and open for 2 minutes at room temperature.
9. Dissolve the pellet in 150 µl of TE buffer and incubated at 37°C for 1 hour and store at -20°C until for future use in PCR amplification.

Appendix 1B: High Pure PCR Template Preparation (Roche) Kit

Materials

1. Lysis buffer (Roche)

2. Proteinase K (Roche)

3. Binding buffer (Roche)

4. Isopropanol (Merck)

5. Wash buffer (Roche)

6. Elution buffer (Roche)

7. High Pure Filter Tubes into one Collection Tubes (Roche)

8. 1.5 ml Eppendorf tubes (WhiteHead Scientific)

9. Filtered 1000 µl, 100 µl and 10 µl pipettes (WhiteHead Scientific)

10. Heating block

11. Vortex

12. Centrifuge

Method

1. Add 200 µl of sample material, 200 µl Binding Buffer and 40 µl Proteinase K to a nuclease-free 1.5 ml microcentrifuge tube Mix immediately and incubate at +70°C for 10 minutes.

2. Add 100 µl Isopropanol and mix well.

3. Insert one High Pure Filter Tube into one Collection Tube and Pipet the sample into the upper buffer reservoir of the Filter Tube and centrifuge 1 minute at 8,000 × g.

4. Remove the Filter Tube from the Collection Tube; discard the flow through and the Collection Tube and Combine the Filter Tube with a new Collection Tube and Add 500

µl Inhibitor Removal Buffer to the upper reservoir of the Filter Tube. Centrifuge 1 minute at 8,000 × g.

5. Remove the Filter Tube from the Collection Tube; discard the flow through and the Collection Tube and Combine the Filter Tube with a new Collection Tube. Add 500 µl Wash Buffer to the upper reservoir of the Filter Tube and centrifuge 1 minute at 8,000 × g and discard the flow through.
6. Remove the Filter Tube from the Collection Tube; discard the flow through and the Collection Tube and Combine the Filter Tube with a new Collection Tube. Add 500 µl Wash Buffer to the upper reservoir of the Filter Tube and centrifuge 1 minute at 8,000 × g and discard the flow through.
7. Centrifuge the entire High Pure assembly for an additional 10 seconds at full speed and discard the Collection Tube.
8. Insert the Filter Tube into a clean, sterile 1.5 ml microcentrifuge tube and add 200 µl prewarmed Elution Buffer to the upper reservoir of the Filter Tube. Centrifuge the tube assembly for 1 minute at 8,000 × g.

Appendix 1C: QIAamp DNA Mini kit (Qiagen)

Materials

1. ATL (tissue lysis buffer) (Qiagen)
2. Buffer AL
3. Proteinase K (Qiagen)
4. Ethanol (Merck)
5. Buffer AW1 (wash buffer 1) (Qiagen)
6. Buffer AW2 (wash buffer 2) (Qiagen)
7. AE (elution buffer) (Qiagen)
8. DNeasy Mini spin columns and 2 ml collection tubes (Qiagen)

9. 1.5 ml Eppendorf tubes (WhiteHead Scientific)

10. Filtered 1000 µl, 100 µl and 10 µl pipettes (WhiteHead Scientific)

11. Heating block

13. Vortex

13. Centrifuge

Method

1. Place small pieces of cysts in 180 µl of ATL (tissue lysis buffer).

2. a Add 20 µl of the commercial Proteinase K in cyst samples and mix.

2. b Pipet 20 µl Proteinase K into the bottom of a 1.5 ml microcentrifuge tube then add 200 µl of blood sample

3. Incubate only the tubes with cyst samples at 56°C until the cells are completely lysed.

4. Add 200 µl of Lysis buffer AL Buffer in both blood and cyst samples and mix by pulse-vortexing for 15 seconds, and incubate cyst samples at +70°C and blood samples at 56°C for 10 minutes. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

5. Add 200 µl of ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

6. Apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

7. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 minutes.

9. Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.

10. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 minute.

11. Repeat step 10.

APPENDIX 2

CONVENTIONAL PCR TECHNIQUES

Materials

1. My*Taq*™ HS Mix PCR kit

2. *T. saginata*: Tsag-*cox*1 Primers
   - Forward Primer F3 5’-TCGGCAAATATTTAATTCCTTTG-3’
   - Reverse Primer B3 5’-AAATTCTAGACGCACCCG-3’
3. *T. solium*: Tsol-*cox*1 Primers
   - Forward Primer F3 5’-CCTATTTTAATTGGAGGTTTTGG-3’
   - Reverse Primer B3 5’-CTACCCCACTTCCTCTTGA-3’
4. Nuclease-free water

5. TE (1mM Tris, 0.1mM EDTA, pH 8.0) buffer

6. 1.5 ml Eppendorf tubes and 0.2 ml PCR tubes (WhiteHead Scientific)

7. Filtered 100 µl and 10 µl pipettes (WhiteHead Scientific)

8. Vortex

9. Centrifuge

Orders from Integrated DNA Technologies

Primer and probe dilution

Primers:

1. Primers will be provided as 10 nmol pure powder.

2. Quickly spin down the primer tube to make sure that powder is on the botttom.

3. Dissolve the powder in 100 µl nuclease-free water or TE (1mM Tris, 0.1mM EDTA, pH 8.0) buffer to get 100 µM probe and store at -4°C.

My*Taq*™ HS Mix PCR reagent kit assembly

My*Taq*™ HS Mix PCR kit

Kit Name: My*Taq*™ HS Mix PCR

Catalogue Number: BIO-25045

Quantity: The kit designed for 200 reactions

Product Information:

- My*Taq*™ HS Mix is a ready-to-use 2x mix for fast, highly-specific, hot-start PCR.
- Allows fast cycling conditions to be used, greatly reducing the reaction time without compromising PCR specificity and yield.
- Contains all the reagents including My*Taq* buffer, dNTPs, MgCl2, enhancers and stabilizers necessary for trouble-free PCR reaction set up.

Recommended Use:

- My*Taq*™ HS Mix enables success in multiplex PCR, colony PCR, genotyping, Low-copy PCR assays, and fast PCR reactions.

PCR mastermix for the amplification of 238 bp and 211 bp *cox*1 genes in a total of 25 µl using My *Taq* PCR assay

Reaction mix Components	**Volume/reaction µl**
Buffer 1x	12.5 µl
Template	2 µl
Reverse Primer	1 µl
Forward Primer	1 µl
Distilled water	10 µl
Total reaction volume	**25 µl**

Thermal Cycler Conditions: (Labnet MultiGene thermal-cycler)

Steps	**Cycles**	**Time**	**Temperature**
initial denaturation	1 cycle	5 minutes	94°C
Amplification			
Denaturation	35 cycles	10 seconds	94°C
Annealing		30 seconds	58°C
Extension		30 seconds	72°C
Final extension		10 minutes	72°C
Cooling	1 cycle	10 seconds	4°C

APPENDIX 3

AGAROSE GEL ELECTROPHORESIS

Materials

1. Agarose (SEAKEM® LE agarose; FMC Bio Products).
2. 50X TAE buffer (Tris Acetate EDTA, pH 8.0).
3. Ethidium bromide (Promega)
4. DNA ladder (100 bp) (Promega)
5. 6x loading dye (Promega)
6. Gel casting plate.
7. Gel sealing tape
8. Buffer tank.

Method

1. Make 1000 ml of 1x TAE, by measuring 20 ml of 50x stock into a 1000 ml graduated cylinder and fill up to 1000 ml with distilled water.
2. Weigh 2.0 g of agarose powder (for 2% gel) and dissolve it in 100 ml of 1x TAE. Melt agarose solution in microwave until completely dissolved. Let stand at room temperature to cool.
3. Prepare gel-casting plate by placing the sample comb in the proper position on the gel tray.
4. Pour 100 ml of 2% agarose on to the gel casting tray slowly and allow it to solidify. After gel has set, final gelling carried out at 4 °C for 30 minutes. There should not be any air bubbles in the gel.
5. Remove the tapes and keep the gel plate in the buffer tank. Add 1x TAE buffer into the reservoirs until it covers the surface of gel at a depth of 3-4 mm.

6. Slowly remove the sample comb and load the samples into the wells (Mix 1-20 µl of RNA or DNA with 6x gel loading buffer; loading volume depends on the sample and make sure gel loading buffer get diluted to 1x with sample).

7. Voltage 100V for 30 minutes or until the first band has migrated at least 2.5 inches away from the wells. The voltage must never exceed 100V. Always run the gel in constant voltage.

8. Stain gel in ethidium bromide, letting it sit for 5-10 min. Distain gel by placing in deionized distilled water for 5 min.

9. Transfer the gel onto a UV light source. Usually a trans-illuminator is used to facilitate this step. Place a piece of plastic wrap between the gel and surface of the illuminator. Use a face shield, gloves, and body shield to minimize the UV exposure. Take a picture for records.

APPENDIX 4

REAL-TIME-PCR TECHNIQUES

Materials

1. 2x LightCycler® 480 Probe Master Kit

2. *T. saginata*: Primers and probe
   - Forward Primer: *Saginata* F- 5’-TTCggCAAATATTTAATTCCTTTg-3’
   - Reverse Primer: *Saginata* R- 5’-AAATTCTAgACgCACCCg-3’
   - Probe: *Saginata* TM- 6FAM-AAAAgTCCACCCTATACCAgCACC--BBQ
3. *T. solium*: Primers and probe
   - Forward Primer: *Solium* F- 5’-CCTATTTTAATTggAggTTTTgg-3’
   - Reverse Primer: *Solium* R- 5’-AACATTAAAAAATCTACCCCACTTCC-3’
   - Probe: *Solium* TM- 6FAM- TgCATggTTGTTAgTTCCATCAATTgTTT -BBQ
4. Nuclease-free water

5. LightCycler® 480 Probe Master Water PCR-grade

6. LightCycler® 480 multiwell plate 96 (Roche)

7. TE (1 mM Tris, 0.1mM EDTA, pH 8.0) buffer

8. 1.5 ml Eppendorf tubes and 0.2ml PCR tubes (WhiteHead Scientific)

9. Filtered 100 µl and 10 µl pipettes (WhiteHead Scientific)

10. Vortex

11. Centrifuge

Primer and probe dilution

Orders from TIB-MOLBIOL

Primers:

1. Quickly spin down the primer tube to make sure that powder is on the botttom.

2. Add sterile nuclease-free water to dissolve the powder and store at -4°C.

Probes:

5. Spin down the probe tube to remove drops from the inside of the lid.

6. Add TE buffer (1mM Tris, 0.1mM EDTA, pH 8.0) to dissolve the powder and store at -4°C.

LightCycler® 480 Probes Master real-time PCR reagent kit assembly

LightCycler® 480 Probes Master kit

Kit Name: LightCycler® 480 Probes Master

Catalogue Number: 04 887 301 001

Quantity: The kit designed for 500 reactions

Product Information: -10 vials of 5 ml of LightCycler® 480 Probes Master, 2x conc

-2 vials of LightCycler® 480 Probes Master, water PCR-grade

- Store the kit at -15 to -25°C

Recommended Use: LightCycler® 480 Probes Master is designed for research studies is a ready to use hot-start reaction mix designed specifically for detecting DNA targets with hydrolysis probes.

Reaction setup of LightCycler® 480 Probes Master real-time PCR mix.

Reagents	**Volume per reaction**
2x LightCycler® 480 Probe Master reaction mix	10 µl
Forward primer	2 µl
Reverse primer	2 µl
*Taq*Man probe	0.8 µl
LightCycler® 480 Probe Master water PCR-grade	0.2 µl
template DNA	5 µl
Total	**20 µl**

Probe setting:

Reporter Dye: 6-carboxyfluorescein (FAM)

Quencher Dye: N'-tetramethyl-6-carboxyrhodamine (BBQ) fluorescent quencher

Thermal Cycler Conditions: (LightCycler® 96 Real-time PCR machine)

Three-step thermal-cycling conditions of real-time *Taq*Man hydrolysis probe.

Program Name	**Cycles**	**Analysis Mode**
Pre-incubation	1	None
3 step Amplification	45	Quantification
Cooling	1	None

Program	**Temp °C**	**Hold**	**Ramp/rate °C**	**Acquisition Mode**
Pre incubation	95ºC	10 min	4.4	None
Amplification	95ºC	10 sec	4.4	None
	58ºC	30 sec	2.2	None
	72ºC	30 sec	4.4	Single
Cooling	40ºC	10 sec	2.2	None

APPENDIX 5

MONOCLONAL HP10 ANTIGEN DETECTION ELISA

Materials

1. 2x 50ml McAbHP10 5mg/ml.

2. 2x 250µl McAb HP10-Biotin conjugate at 2.5 mg/ml.

3. 30 ml positive control

4. 30 ml negative control

5. 500 µg Streptavidin Peroxidase conjugate Sigma S-5512

6. Bovine serum albumin (A-4503)

7. Phosphate buffered saline tablets pH 7.3

8. Carbonate buffer tablets (C3041)

9. Immulon 1 flat bottomed 96 well ELISA plates Thermo Life Science Ltd

10. 100µl and 10µl pipettes (WhiteHead Scientific)

11. Multichannel pipette

12. 120 ml flask

13. Tetramethylbenzidine Substrate Sigma T-8665

14. 0.2 M H_2SO_4

15. Mixing boats

Preparation of reagents

1. McAb-HP10 (5mg/ml) 0.5 ml lyophalised.

- Add 0.5 ml distilled water to the vial and dissolve the lyophalised McAb. Prepare 20 µl aliquots and store frozen at -20 ºC. Use 10 µg/ml to dissolve in the coating buffer.

2. McAb-HP10 Biotin (2.5 mg/ml) 250 µl lyophalised.

- Add 250 µl pure distilled water to the vial and dissolve the lyophalised McAb Biotin conjugate. Prepare 20 µl aliquots and store frozen at -20 ºC. Once defrosted store the aliquot at 4 ºC until it is finished. Avoid freezing and thawing of the conjugate.

3. Control serum (1 ml aliquots)

- 1 ml aliquots are stored frozen at -20 ºC until use. Use the undiluted at 100 µl per well.

4. Streptavidin Peroxidase conjugate Sigma lyophalised 500 µg

- Add 500 µl pure distilled water to the vial and dissolve the lyophalised conjugate. Prepare 20 µl aliquots and store frozen at -20 ºC. Once defrosted store the aliquot at 4 ºC until it is finished. Avoid freezing and thawing of the conjugate.

5. Washing solution – 0.9 %(w/v) NaCl-0.05% (w/v) Tween20

- Dissolve 375 µl of Tween 20 into 450ml of PBS.

6. PBS/BSA/Tween (Blocking solution, diluent and for background estimation wells)

- **Phosphate Buffered Saline pH 7.3 (PBS) solution using the Sigma tablet**
  - Dissolve 1 tablet into 100 ml of distilled water.
- **PBS/BSA/Tween (Blocking solution)**
  - Add 250 µl of Tween 20 into 450ml of PBS.
  - Add 5 g of BSA and leave the BSA to dissolve slowly into the PBS/Tween and make the solution up to 1 litre with PBS.
  - Prepare 25 ml aliquots in plastic universal bottles and store at -20 ºC.
- **Coating buffer**
  - Dissolve 1 Bicarbonate buffer capsule into 100 ml of distilled water.

- **Stop solution**
  - Add 2 ml of H_2SO_4 into 100 ml of distilled water.

Method

Preparation for 1 plate

1. Dissolved 2.5µl of HP10 into 10ml of prepared coating buffer then incubate at 4°C overnight.

2. Wash Plate twice with the washing solution.
3. Block the non-reacted antibodies by adding 200µl of blocking solution to each well, means the antibodies that did not bind to the plate are prevented from further binding to the plate.
4. Wash plate three times with washing solution.
5. Add undiluted 50µl serum to each well.
6. Wash plate three times with the washing solution.
7. Dissolve 40µl of biotinylated antigen (HP10) in 10ml of PBS/BSA/Tween solution in the mixing boat then transfer 100µl of the solution to each well.
8. Wash plates three times with the washing solution.
9. Dissolve 1µl of Streptavidin peroxidase in 10ml of PBS/BSA/Tween solution then transfer 100µl of the solution was to each well.
10. Wash plate three times with the washing solution.
11. Add 100µl of TMB (tetramethylbenzidine) to each well.
12. Add 100µl of 0.2M H_2SO_4 to each well to stop the reactions.
13. Read the optical densities of the plate at wavelength of 450nm on an ELISA plate reader

ELISA plates were routinely set up to include five positive, five negative and six diluent control wells (Figure 4.1 A), and each test sample was run in duplicate. The mean sample ODs minus the mean diluent ODs were corrected for any day to day variation using a correction factor determined by the formula:

Correction Factor = Mean P^0 - Mean N^0/ Mean P^t - Mean N^t

where P = positive control

N = negative control

0 is the reference day

t is the test day.

ELISA results are rejected if the correction factor for any particular plate varied more than 10% from the reference day. Samples are run with different reagents and positive and negative controls; hence the negative cut off point is determined on a plate to plate basis using the formula: 2X + 3sd of negative controls, where X = the mean of the negative control and sd = standard deviation from the mean of the negative control.