Development of high resolution melt qPCR assays for detection of *Toxoplasma gondii*, *Cryptosporidium* and *Trypanosoma* species

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µl- microliter

µm- micrometer

µM- micromolar

AAT- animal African trypanosomiasis

ag/µl- attogram per microgram

Aids- Acquired immunodeficiency syndrome

ALFP- Amplified fragment length polymorphism

B1 gene- B1 protein

bp- base pairs

BCT- buffy coat technique

BLAST- Basic Local Alignment Search Tool

Bst DNA polymerase- *Bacillus stearothermophilus* deoxyribonucleic acid polymerase

C1- Repressor protein C1 gene

C. parvum- Cryptosporidium parvum

C. hominis- Cryptosporidium hominis

C. erinacei- Cryptosporidium erinacei

C. meleagridis- Cryptosporidium meleagridis

CATT- Card-Agglutination Trypanosomiasis Test

Cell/ml- cell per milliliter

COWP- Cryptosporidium oocyst wall protein

CT values- cycle threshold values

DFA- Direct immunofluorescence assay

DHF gene- Di-hydrofolate

DNA- Deoxyribonucleic acid

dNTPs- dinucleotide triphosphates

DT- Sabin-Feldman Dye Test

EDTA- Ethylenediaminetetraacetic acid

EIA- Antigen-capture-based enzyme immunoassay

ELISA- Enzyme-linked immunosorbent assay

et al.- et alia

fg/µl- femtogram per microlitre

FITC- Fluorescein isothiocyanate

GC-content - guanine-cytosine content

GP40/15- glycoprotein gp40/15

GP60-60-kDa glycoprotein

HAT- human African trypanosomiasis

HCT- Haematocrit centrifugation technique

HIV-Human immunideficiency virus

HSP70- 70-kDa heat shock protein

HRM- High resolution melt

HRM-qPCR- High resolution melt-quantitative polymerase chain reaction

IAAT- Immunosorbent agglutination assay test

ICT- Immunochromatographic test

IFA- Immunofluorescence assays

IFAT- Indirect fluorescent antibody test

IgG- Immunoglobin G

IgM- Immunoglobin M

IHA- Indirect hemagglutination assay

Inc.- Incorporated

ISAGA- Immunosorbent agglutination test

ITS-1- Internal transcribed spacer

kDa- kilodaltons

LAMP- Loop-mediated isothermal amplification

LAT- Latex agglutination test

M- Molar

mAECT- Miniature anion exchange columns

MAS-PCT- Multiplex allele-specific polymerase chain reaction

MAT- Modified agglutination tests

ml- millilitre

MLST- Multilocus sequence typing

mM- millimolar

NaCl- Sodium Chloride

NCBI- National Center for Biotechnical Information

ng/µl -nanogram per microlitre

NWU- North-West University

PCR- Polymerase chain reaction

PFR- Paraflagellar rod protein

PIA- Piezoelectric immunoagglutination assay

Propan-2-ol-Isopropyl alcohol

qPCR- quantitative polymerase chain reaction

RAPD- Randomly amplified polymorphic deoxyribonucleic acid

RFLPs - Restriction enzyme fragment length polymorphisms

rpm- Revolution per minute

rP0- Ribosomal protein P0

RIME- Repetitive insertion mobile element

RNA- Ribonucleic acid

rRNA- Ribosomal ribonucleic acid

s.- seconds

spp. -Species

SRA- Serum resistance associated

T. brucei- Trypanosoma brucei

T. b. brucei- Trypanosoma brucei brucei

T. b. gambiense- Trypanosoma brucei gambiense

T. b. rhodesiense- Trypanosoma brucei rhodesiense

T. congolense- Trypanosoma congolense

T. simiae- Trypanosoma simiae

T. godfreyi- Trypanosoma godfreyi

T. evansi- Trypanosoma evansi

T. equiperdum- Trypanosoma equiperdum

T. gondii- Toxoplasma gondii

T. vivax- Trypanosoma vivax

Taq polymerase- *Thermus aquaticus* thermostable DNA polymerase

TgsGP- Trypanosoma brucei gambiense specific glycoprotein

T_m- Primer melting temperature

TRAP gene- Triiodothyronine Receptor Auxiliary Protein

Tris-HCl – Trisaminomethane-Hydrochloric acid

USA- United States of America

ve- - negative control

ve+- positive control

WB- Western blotting

WHO- World Health Organization

RESEARCH OUTPUTS

Conference papers

F. Coetzee, O. Thekisoe, F. Taute & E. Suleman. The development of a colorimetric nanoparticle assay for diagnosis of African trypanosomiasis. The 48th Annual of the Parasitological Society of Southern Africa (PARSA) Conference, 15-17 September 2019, Hotel Safari, Windhoek, Namibia. Page 48.

F. Coetzee, O. Thekisoe, F. Taute & E. Suleman. Development of a multiplex HRM-qPCR assay for the detection of *Toxoplasma gondii*, *Cryptosporidium* and *Trypanosoma* species. The 10th Annual National Zoological Gardens Research Symposium. 20-22 November 2019. National Zoological Gardens, Pretoria, South Africa. Page 42.

ABSTRACT

Protozoan parasites are amongst the leading diseases causing agents in both animals and humans. Trypanosomosis, cryptosporidiosis and toxoplasmosis are diseases of medical, veterinary and economic importance caused by *Trypanosoma* spp., *Cryptosporidium* spp. and *Toxoplasma gondii* respectively. Diagnosis is the first line of defence in combatting diseases hence the need for reliable, rapid, specific and sensitive diagnostic assays. Therefore, the aim of this study was to develop a high resolution melt real-time PCR (HRM-qPCR) assay for the detection of *Trypanosoma* spp., *Toxoplasma gondii* and *Cryptosporidium* spp. infections. The HRM-qPCR primers were designed from RIME, rP0, HSP70 and B1 genes of subgenus *Trypanozoon* species, *T. congolense*, *Cryptosporidium* spp. and *Toxoplasma gondii* respectively. The melt curve temperatures were 85°C for the RIME gene, ± 83°C for rP0 gene, 76°C for HSP70 gene and 81°C for B1 HRM-qPCR assays developed in this study. All the assays detected serially diluted DNA of the respective parasites down to 100 ag/ul which is equivalent to less than 1 *Trypanosoma* cell or *Cryptosporidium* or *T. gondii* oocyst per millilitre with all reaction conducted at 60°C for 40 cycles. The HRM-qPCR has showed higher detection sensitivity than conventional PCR for detection of *Cryptosporidium* spp., *Trypanozoon* spp, *Trypanosoma congolense, Toxoplasma gondi* from field derived samples. This newly developed HRM-qPCR assay requires validation with large samples from the field. Following standardization and validation the assays have potential to be used for diagnosis of subgenus *Trypanozoon* spp., *Trypanosoma* spp., *Toxoplasma gondii* and *Cryptosporidium* spp. infections.

Keywords: *Toxoplasma gondii, Trypanosoma* spp., *Cryptosporidium* spp., HRM-qPCR, Diagnostics.

CHAPTER 1

INTRODUCTION

1.1. Background

Protozoan parasites are defined as organisms that are dependent on other organisms for survival and development (Perry & Randolph, 1999). Parasitism is where “the parasite relies upon the host for its nutrients and a place to live and there is potentially some cost to the host” like a disease (Wiser, 2011). Some of the important parasitic protozoans will be looked at in this study.

Trypanosoma spp. are protozoan parasites distributed in Africa, Asia, Europe and South America causing sleeping sickness and Chagas disease in humans, and Nagana, Dourine and Surra in animals (Taylor & Aunthie, 2004). In affected areas the parasite infects the host and is fatal if left untreated (Matthews, 2005). As a result, trypanosomes contribute to poverty and have an impact on the agriculture and development in these areas. According to the World Health Organisation, trypanosomes causes more fatalities surpassing HIV/Aids in these afflicted areas (WHO, 2013).

Toxoplasma gondii, is a parasitic protozoan causing a disease called toxoplasmosis in humans with worldwide distribution (Ramzan *et al*., 2009) and it infects virtually all warm-blooded organisms (Barrat *et al*., 2010). Statistics support the speculation that one third of the human population is infected, being mostly benign. It is dangerous in immunocompromised or pregnant hosts since *T. gondii* can cross the placental-uterine barrier. A study indicated that human congenital *Toxoplasma* transmissions is 19.8% (Barrat *et al*., 2010). Toxoplasmosis can cause severe economic loss due to the high percentage of abortions especially for small ruminant breeders (Ramzan *et al*., 2009). The health impact is also rising due to ingestion of contaminated food, water and milk resulting in infections.

Cryptosporodium is a protozoan parasite causing a disease called cryptosporidiosis which is distributed worldwide (Mayer & Palmer, 1996). Cryptosporidiosis is mostly caused by *C. parvum* and *C. hominis* in humans (Stark *et al*.,2007). Recent studies and statistics indicate an increase in waterborne disease outbreaks by *Cryptosporidium parvum*. The World Health Organisation has labelled it as a neglected pathogen. It is especially dangerous for patients with immunocompromised systems where the disease can become chronic and result in death (Chalmers & Davies, 2010). There is a lack of methodology for the detection of *Cryptosporidium* causing it to make a severe impact in economic loss especially with

neonatal diarrhoea in livestock compromising agricultural development (Smith *et al*., 2007). Another concern is that wildlife infections show adapted species which could potentially be infectious to humans and other animals (Smith *et al*., 2007).

High-resolution melt (HRM) assays was introduced as a technique developed for single nucleotide polymorphism genotyping (Wojdacz & Dobrovic, 2007). Studies have shown that it is possible to use as an application for diagnosing diseases (Malentacchi *et al*., 2009). Quantitative real-time PCR (qPCR) assays provide a sensitive tool for diagnosing and quantifying parasitic infections" (Rojas *et al*., 2017). Fast, reliable diagnosis is made when high-resolution melt analysis is done with qPCR, making it possible to distinguish between DNA amplicons because of different melting temperatures (Rojas *et al*., 2017).

1.2. Problem statement

Protozoan parasites are organisms responsible for various diseases of veterinary, medical and economical importance (Laohasinnarong *et al.,* 2011). Parasitic diseases have a severe economic and health impact worldwide especially in developing countries (Custodio, 2016). The control of the diseases relies on the detection of these parasites and is made possible through reliable diagnostics (Njiru *et al*., 2008). In developing countries medical care is usually very far, making it frequently too expensive for the average household and is mostly seen as a last resort where it is then too late for the care needed. (Smith *et al*., 2015). Productivity in livestock and in the agricultural sector gets decreased (Nappi & Vass, 2002). There is a considerable need for improved epidemiology, accurate diagnostics and vaccine development (Custodio, 2016). Therefore, it is important to develop new and updated diagnostic assays. Diagnostics used-up to date includes a variety of assays with differences between genus, but the predominantly used assay for protozoan parasites is conventional PCR according to Iseki *et al*. (2010). Conventional PCR used along with sequencing is sensitive and specific, but it is laborious and expensive and it requires trained staff to perform experiments(Winder *et al*., 2011). The assay also needs precise instruments and visualisation equipment to show and analyse the results (Njiru *et al*., 2008). Whereas real-time PCR (q-PCR) uses a fluorescent dye for accurate detection and identification that is highly sensitive and minimizes potential experimental errors but includes the same limitations that it is complex and expensive (Winder *et al*., 2011).

Diagnosis is the first line of defence in combatting these infections caused by these diseases hence the need for new and updated diagnostic assays. Therefore, this study focuses on adapting HRM-qPCR for *Trypanosoma* spp., *Toxoplasma gondii* and *Cryptosporidium* spp.

1.3 Aim, objectives and hypothesis

1.3.1. Aim

The aim of this study is to develop a high resolution melt qPCR for the detection of *Toxoplasma gondii, Cryptosporidium* and *Trypanosoma* species.

1.3.2. Objectives

- To design HRM-qPCR primers specific for amplification of *Cryptosporidium* spp., *Toxoplasma gondii* and *Trypanosoma* spp.
- To determine the detection limit of *Cryptosporidium* spp., *Toxoplasma gondii* and *Trypanosoma* spp. HRM-qPCR and assess their potential to amplify DNA from field derived samples.

1.4. Outline of dissertation

Chapter 1 - Introduction

A general introduction where the aim, objectives and problem statement will be discussed.

Chapter 2 - Literature review

This chapter reviews all the parasite species and diseases that are part of this study including details of the background of the parasites, life cycles and the diseases caused. It also includes diagnostics already available and the use of HRM-qPCR.

Chapter 3 - Materials and Methods

This chapter describes the identification of target genes, sample collection, materials used and methods followed, as well as how data is analysed.

Chapter 4 - Results

A presentation of the data obtained in this study.

Chapter 5 - Discussion, conclusion and recommendations

Interpretation of data with conclusion indicating whether the aims and objectives have been achieved. Recommendation for future studies that need to be undertaken with reference to data obtained from this study.

CHAPTER 2

LITERATURE REVIEW

2.1. Trypanosomiasis

The *Trypanosoma* parasites cause human African trypanosomiasis and animal African trypanosomiasis which are diseases endemic in sub-Saharan Africa and continues to be a major health and economic problem in Africa (Bonnet *et al.*, 2015; Buscher *et al.*, 2014). *Trypanosoma* spp. are found in the bloodstream or tissue of animals and humans in various regions throughout the world. The vector spreading the disease to humans and animals in Africa, is the tsetse fly (Genus: *Glossina*) and is considered as the parasite's intermediate host (Troncy *et al*., 1989). There are two major groups of *Trypanosoma* spp. based on different transformation processes namely Salivaria and Stercoraria. The Salivaria group consists of subgenera of *Duttonella*, *Nannomonas, Pycomonas* and *Trypanozoon* which are considered to have the most significant pathogenic *Trypanosoma* spp. The group includes all trypanosomes that are transmitted through a process known as the anterior station development in the tsetse vector. The process includes the multiplication in the digestive tract and proboscis to successfully transmit infection when feeding occurs (Urquhart *et al*., 1987). Stercoraria group consists of subgenera *Herpetosoma* and *Schizotrypanum* (Stevens & Brisse, 2004). In this group, transmission occurs through the faeces of the insect vector after the synthesis of infective metatrypanosomes in the digestive tract (Uilenberg, 1998).

2.1.1. Classification of trypanosome parasites

The classification of *Trypanosoma* according to (Sherwood *et al*., 2014; Stevens & Brisse, 2004) is as follows:

Kingdom: Protista

Phylum: Sarcomastigophora

Class: Zoomastigophorea

Order: Kinetoplastida

Family: Trypanosomatidae

Genus: *Trypanosoma*

Subgenus: *Trypanozoon*

Species: *Trypanosoma evansi*

Trypanosoma equiperdum

Trypanosoma brucei brucei

Trypanosoma brucei gambiense

Trypanosoma brucei rhodesiense

Subgenus: *Duttonella*

Species: *Trypanosoma vivax*

Subgenus: *Herpetosoma*

Species: *Trypanosoma lewisi*

Subgenus: *Nannomonas*

Species: *Trypanosoma congolense*

Trypanosoma simiae

Trypanosoma godfreyi

Subgenus: *Pyconomonas*

Species: *Trypanosma suis*

Subgenus: *Schizotrypanum*

Species: *Trypansoma cruzi*

Trypanosoma theileri

2.1.2. Subgenus *Trypanozoon*

The subgenus *Trypanozoon* includes three species namely. *T. brucei, T. evansi and T. equiperdum,* which are morphologically identical but can be distinguished by pathology, epidemiology and genetic characteristics (Stevens & Brisse, 2004).

The *T. brucei* contains the species most important in Africa. *T. brucei* can be divided into three subspecies namely *T. brucei brucei, T. brucei gambiense* (West Africa) and *T. brucei rhodensiense* (East Africa) (Stevens & Brisse, 2004).

T. brucei brucei causes animal African trypanosomiasis, also called Nagana, in domestic mammals, camels, some antelope and carnivores (Stevens & Brisse, 2004). Nagana is a wasting disease and is often fatal (Connor, 1994). Nagana contains an acute and chronic phase where the acute phase includes symptoms like anaemia, oedema, enlarged spleen

and lymph nodes, abortions, death and increased neonatal mortalities (Taylor & Aunthié, 2004). Other forms of transmission include congenital transmission and the ingestion of infected meat, but research show it can't infect humans (Uilenberg, 1998).

Two forms of human African trypanosomiasis or sleeping sickness can be distinguished due to geographic distributions (Nappi & Vass, 2002). In humans, the chronic form of the disease is *T. b. gambiense* that causes human African trypanosomiasis in eastern Africa as seen in figure 2.1. Most cases reported of the disease is due to *T. b. gambiense*. The *T. b. gambiense* infects humans, sheep, pigs and wild animals (Njiru *et al*., 2008). The lymphoid and nervous system is usually involved with *T. b. gambiens*e and can extend over a period of time (Nappi & Vass, 2002). Symptoms include enlarged lymph nodes, spleen and liver, oedema, anorexia, weight loss, blurred vision, paralysis and meningoencephalitis, coma (Nappi & Vass, 2002). Other symptoms also include headache, fever, malaise, anaemia and urticaria (Harman & Mason, 2002). Other forms of transmission are congenital transmission, blood transfusions and organ transplantation (WHO, 2013).

The other form of human African trypanosomiasis or sleeping sickness is *T. b. rhodesiense* and is distributed mostly in western Africa as seen in figure 2.1 (Simarro *et al*., 2008; Carrington *et al*., 2001). *T. b. rhodesiense* causes the virulent, acute form of the disease and will only last a few months. The nervous system isn't associated with *T. b. rhodesiense* due to the rapid time of infection. Domestic and native game animals serve as a reservoir host for *T. b. rhodesiense* (Nappi & Vass, 2002). Symptoms include headache, fever, oedema, enlarged lymph nodes, thyroid disfunction, adrenal insufficiency and hypogonadism (WHO, 2013). Other symptoms that has been observed is jaundice, hyperbilirubinemia and ascites. Congenital transmission can be included but is highly unlikely (WHO, 2013).

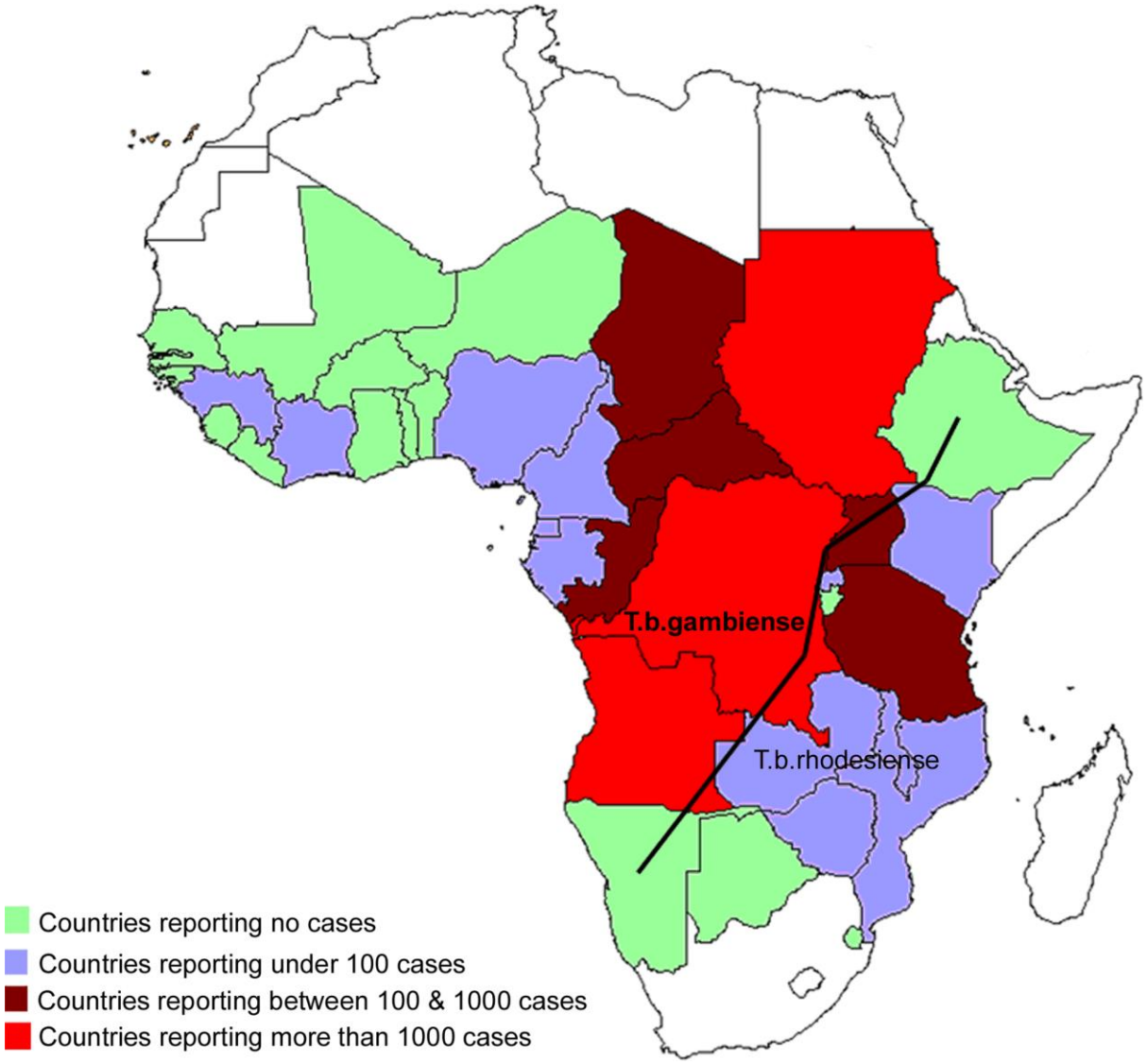

Figure 2.1: Map of Africa showing the epidemiological status of countries considered endemic for the disease (Simarro *et al*., 2008).

The *T. evansi* is thought to be derived from *T. b. brucei* where the kinetoplastic mitochondrial DNA was deleted making this species unable to complete the life cycle in the tsetse fly (Dargantes *et al.*, 2013). *T. evansi* is transmitted through 'mechanical inoculators' mostly tabanid flies (eg. *Tabanus* and *Stomoxys*) and is found worldwide excluding North America and Australia (Stevens & Brisse, 2004). *T. evansi* ranges from 15-36 µm and is monomorphic that only occur in a long and slender form. *T. evansi* causes a disease known as Surra that is a wasting disease in wildlife and domestic livestock. The animals that are most affected is dogs, camels, bovines, equines and water buffalo causing severe economic losses (Dargantes *et al.*, 2013). It can infect vampire bats in Latin America, where the bats can serve as reservoirs or hosts (Hofkin & Loker, 2015).

The *T. equiperdum* is monomorphic and causes dourine in horses, donkeys and mules (equines) (Vulpiani *et al*., 2013). *T. equiperdum* and *T. evans*i cannot be morphologically distinguished from one another (Calistri *et al*., 2013). Dourine has a worldwide geographical distribution but is endemic to Asia, Africa, the Middle East, and eastern Europe (Vulpiani *et al*., 2013). Dourine is a chronic disease of equines that is transmitted during coitus between equine hosts (Clausen *et al*., 2003). Congenital transmission can also happen from mother to foal. The parasite is rarely detected in blood and can be detected in tissue mostly (Taylor & Aunthié. 2004). The symptoms can be divided into three stages (Vulpiani *et al*., 2013). Stage one includes inflammation and oedema of genitalia and genital lesions. Stage two contains cutaneous plaques. The name dourine is derived from these cutaneous plaques that is "circular elevated plaques of thickened skin" (Calistri *et al*., 2013). Stage three include anaemia, involvement of the nervous systems, causing neurological disorders, and will usually lead to death (Vulpiani *et al*., 2013).

2.1.3. Subgenus *Nannomonas*

Included in the subgenus *Nannomonas* is *T. congolense*, *T. simiae* and *T. godfreyi* (Stevens & Brisse, 2004). The morphology of *T. congolense* includes three different strains that differ through length and forms. The three strains have been divided into three groups namely:

- Savanna: Savanna is the dry division ranging over East and West Africa savanna.
- Riverine/forest: Riverine/forest is the humid division.
- Kilifi: Stretching over the Kenya coast.

T. congolense has a wide host range including bovines, equines, pigs, dogs, sheep, camels and goats. *T. congolense* causes animal African trypanosomiasis affecting livestock (Torres *et al*., 2018).

T. simiae causes animal African trypanosomiasis (Isaac *et al*., 2016). *T. simiae* is a rapid, lethal pathogen to domestic pigs (Hamill *et al*., 2013). It is usually fatal disease outbreak that occur in short durations (Stevens & Brisse, 2004).

T. godfreyi also causes animal African trypanosomiasis (Isaac *et al*., 2016). This genotype was first identified in the Gambia and shown to infect pigs (Gibson, 2007). It is widely distributed and have been found over Africa (Masiga *et al.,* 1996). It is a chronic form of the disease and sometimes a lethal infection leading to death (Stevens & Brisse, 2004).

2.1.4. General life cycle of tsetse transmitted *Trypanosoma* spp.

When the insect vector feeds on an infected host, trypanosomes are ingested as seen in figure 2.2 (Nappi & Vass, 2002; Global Health, Division of Parasitic Diseases, 2019). In the digestive tract of the vector the glycoprotein coat disintegrates and transforms into procyclic trypomastigotes for *T. brucei* and *T. congolense* (Urquhart *et al*., 1987). The trypomastigotes become elongated and multiply by binary fission in the midgut transforming into epimastigotes (Matthews *et al*., 2004. With *T. brucei* the epimastigotes migrates to the salivary glands and with *T. congolense* to the proboscis where they will multiply further and differentiate into metacyclic trypomastigotes (Urquhart *et al*., 1987). The metacyclic trypomastigotes (infective stage) are inoculated, through the skin, into the bloodstream of a new host when feeding. The feeding site will usually become inflamed and a chancre will sometimes appear (Uilenberg, 1998). The parasite will proliferate into trypamostigotes that can cause a secondary stage of the disease by entering the circulatory system (WHO, 2013).

The *T. evansi* is very similar to *T. brucei* but includes other flies that are mechanical inoculators. No cyclical development takes place in the insects and the parasite is transmitted mechanically. *T. equiperdum* is directly transmitted through coitus between an infected host and a healthy host and will penetrate the mucous membrane of the reproductive organs that starts the infection (Urquhart *et al*., 1987).

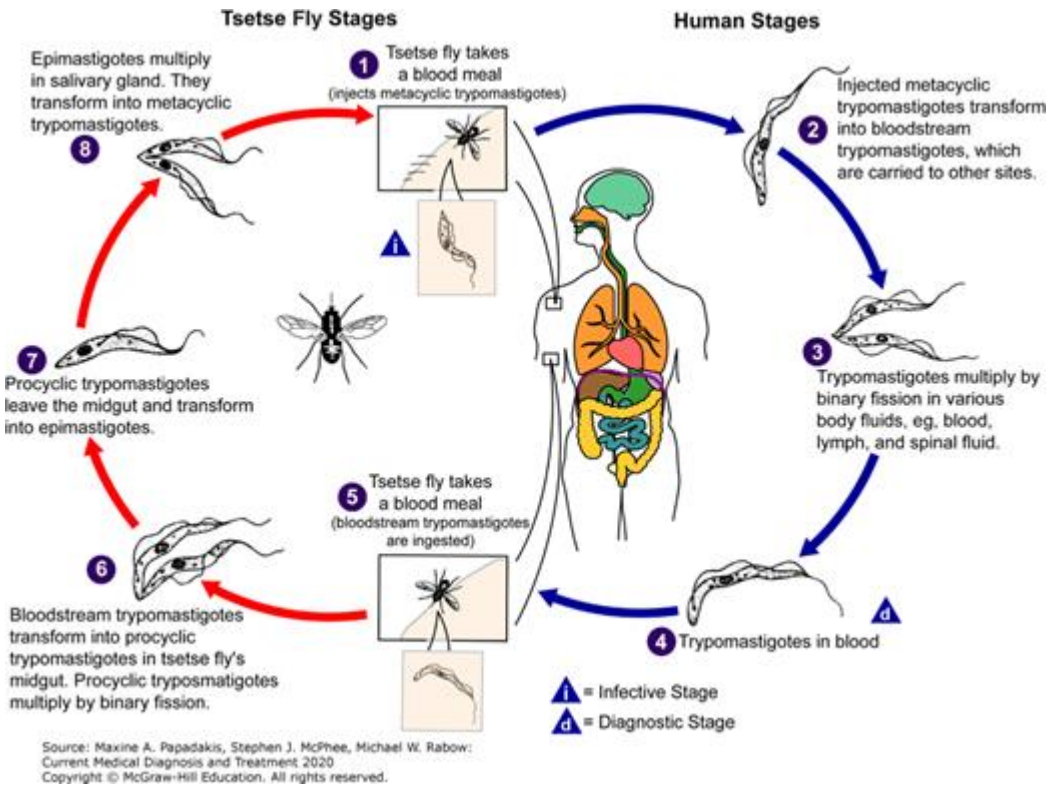

Figure 2.2: Life cycle of trypanosomes (Global Health, Division of Parasitic Diseases, 2019).

2.1.5. Diagnostics

Diagnosis of the infection of human African Trypanosomiasis (HAT) or infection of animal African Trypanosomiasis (AAT) is very important to the community (Buscher & Lejon, 2004). It is used to control and monitor the spread of the diseases and is required to address the epidemiology of the disease and the control strategies (Busher *et al*., 2014).

2.1.5.1. Clinical signs and symptoms

This method is normally used in rural areas through physical examination (Eisler *et al*., 2004). Physical examination is used when diagnostic techniques are not available, or the diagnostic techniques are too expensive. (Leak, 1999). Diagnosis cannot only be based on clinical signs as some are not specific to trypanosomiasis, as a result the presence of the parasite must be confirmed using other methods (Buscher *et al.*, 2014).

2.1.5.2. Microscopic tests

Wet blood films can be analysed by using a microscope to detect mobile trypanosomes. It is a quick technique but is not very sensitive or specific (Nantylya, 1990).

Thin and thick Giemsa stained blood smears, as seen in figure 2.3 (A), can be examined underneath a light microscope (figure 2.3 (B)) to detect trypanosomes in the blood between the other cells (Eisler *et al*., 2004). This method is not sensitive enough to detect the parasite in the earlier stages of infection but is simple (Eisler *et al*., 2004).

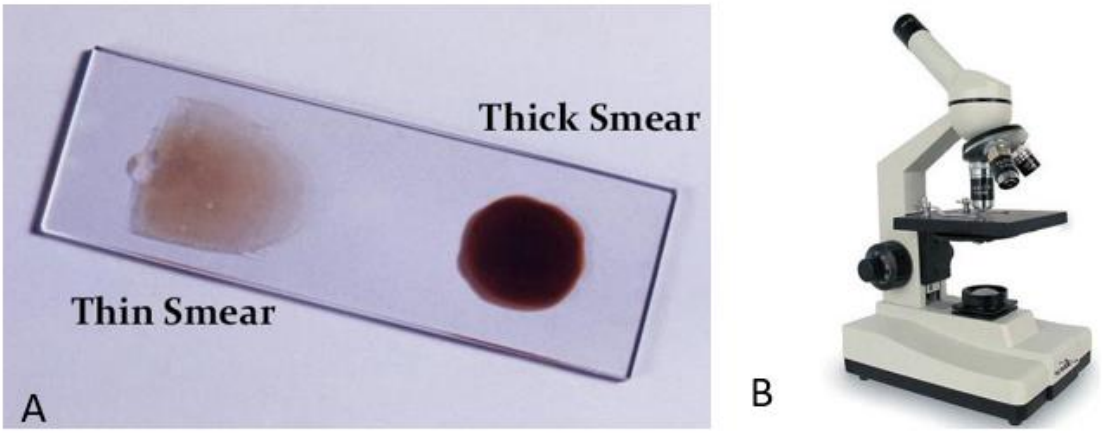

Figure 2.3: Visual illustration of thin and thick Giemsa stained blood smears (A) and a light microscope (B) (Aryal, 2019; School of Biomedical Sciences Wiki,2018).

2.1.5.3. Concentration techniques

Haematocrit centrifugation technique (HCT) (figure 2.4 (A)), the buffy coat technique (BCT) (figure 2.4. (B)) and miniature anion exchange columns (mAECT) (figure 2.4. (C)). The HCT and BCT techniques can be used under field conditions with the necessary equipment. The HCT is also known as the Woo test and uses capillaries with anticoagulant and the blood sample gets centrifuged in a haematocrit centrifuge. The parasite is then concentrated between the plasma and erythrocytes (Busher & Lejon, 2004) but the technique is time consuming (Buscher *et al.*, 2005). The mAECT uses anion chromatography to separate the parasite from the blood cells in a sealed glass tube using low centrifugation (Busher & Lejon, 2004). The parasite cells will be concentrated on the bottom of the glass and can be examined by a microscope. All these methods are very specific but low in sensitivity and is therefore limited when the parasite is low in the samples and are time consuming techniques (Buscher *et al.*, 2014).

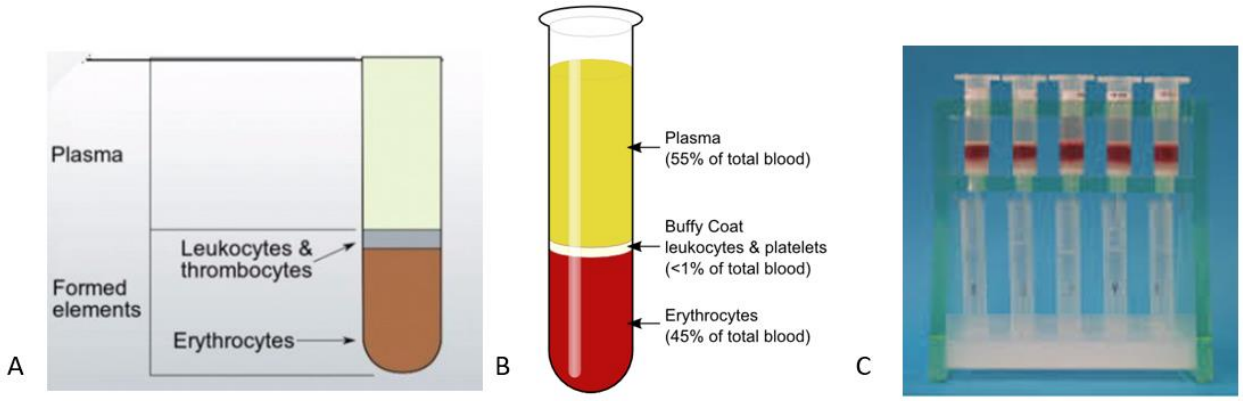

Figure 2.4: Visual illustration of haematocrit centrifugation technique (HCT) (A), the buffy coat technique (BCT) (B), miniature anion exchange columns (mAECT) (C) (Medicalhub, 2014; Wikipedia, 2019; Busher *et al.,* 2009).

2.1.5.4. Serological tests

Card-Agglutination Trypanosomiasis Test (CATT) can be used to test serum or diluted blood for the detection of trypanosomes in suspected cases (figure 2.5) (Bonnet *et al.*, 2015). It is specific for the detection of *T. b. gambiense*-specific antibodies from samples that will agglutinate with the antigen in the reagent (Buscher *et al.*, 2005). A field kit is available but false negative tests are very common especially when the blood is undiluted. This test is high in specificity but is not sensitive in some cases (Buscher *et al.*, 2005).

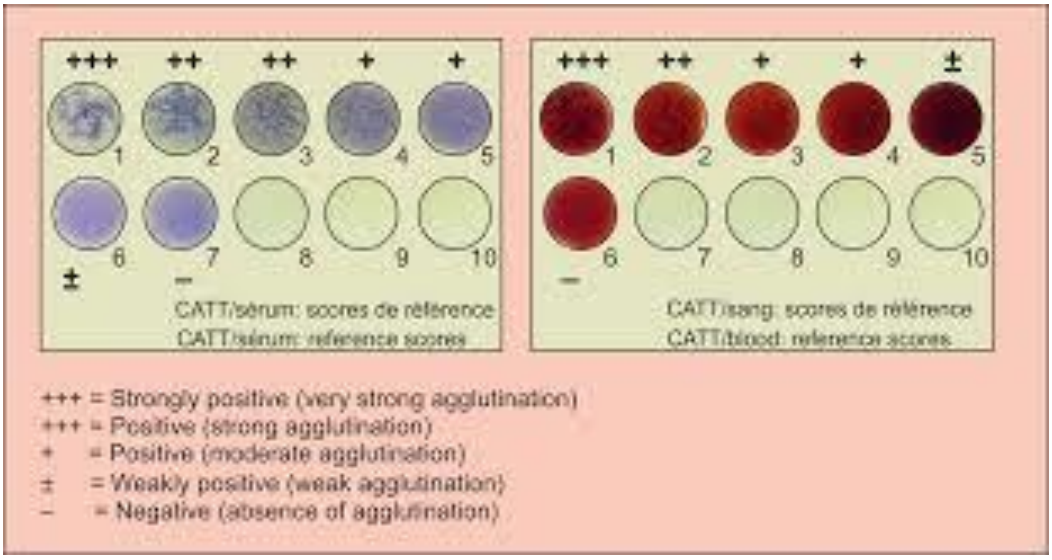

Figure 2.5: Visual illustration of card-Agglutination Trypanosomiasis Test (CATT) (Joshi, 2013).

2.1.5.5. Immunofluorescence assays (IFA)

This test is used widely throughout Africa as it is sensitive and specific but isn't cost effective and can't be applied in the field (Hahon & Cooke, 1967). Serum and blood filter paper samples can be used although impregnated filter paper has shown low signs of sensitivity (Hahon & Cooke, 1967). This assay detects any infection, of humans and animals, for which antibodies are available (Beutner, 1961). Antibodies are conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC) (Beutner, 1961). The antibodies bind to the specific antigens and visualize the antigen appearing green when observed with a light microscope (Beutner, 1961). Direct IFA uses primary antibodies that binds directly to the antigens. Indirect IFA uses secondary antibodies that are labelled and recognizing the primary antibodies that binds to the antigens (Beutner, 1961).

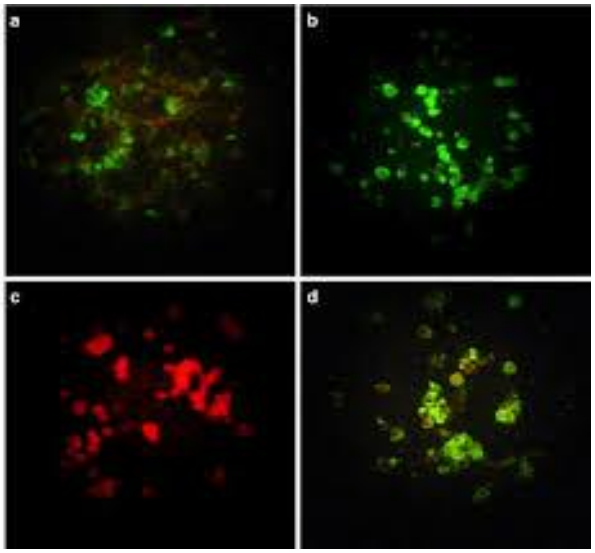

Figure 2.6: Visual illustration of immunofluorescence assays (IFA) (Cabezas *et al*., 2009).

2.1.5.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA can be used on different samples including serum, DNA and blood but it is very time consuming and trained personnel is necessary for the results to be interpreted (Naot *et al.*, 1980). ELISA can be used to identify positive samples present, and the principle of ELISA is that it uses antibodies attached to enzymes (figure 2.7). The antibodies are specific and gets added to the sample containing antigens. The antibodies attach to the antigens if the infection is present and results in a colour change in the substrate (Stopa & Yolken, 1979). A variety of tests can be done using ELISA namely direct, indirect, capture and competitive (Ramirez *et al*., 2017). All the variants are the same for the determination of antigens “except the indirect method that only detects antibodies” (Ramirez *et al*., 2017). ELISA needs antibodies and a limitation is that it gives false-positives and false-negatives.

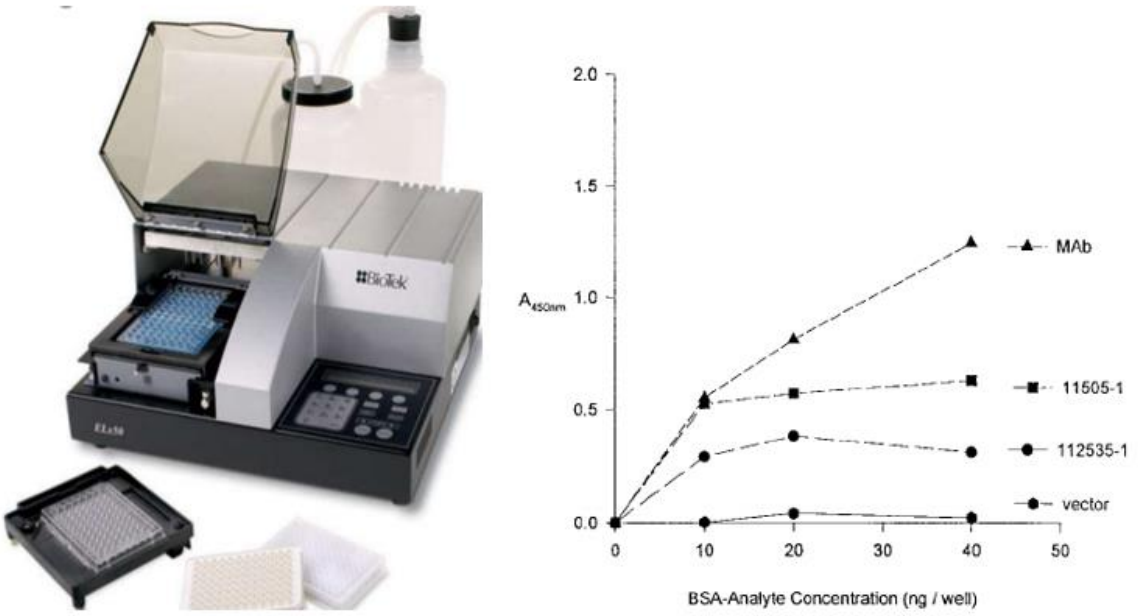

Figure 2.7: Visual illustration enzyme-linked immunosorbent assay (ELISA) (Ahmed, 2015; Ogunjimi *et al*., 1999).

2.1.5.7. DNA-based diagnostic methods

2.1.5.7. (a) Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) amplifies large amounts of DNA using four or six specific primers and *Bst* DNA polymerase under isothermal conditions (Notomi *et al*., 2000). The DNA that is targeted is recognized at six regions by the primers (Parida *et al*., 2008). This method is very specific and simple method in a single step under one hour, using one constant temperature (Mori *et al*., 2001). The product of this technique can be seen visually due to the magnesium pyrophosphate that is formed (figure 2.8) *(*Notomi *et al*., 2000). Kuboki *et al.* (2003) indicated that the sequences, of *T. brucei* PFR A target gene and *T. congolense* P0 target gene, produced stem loop DNAs with inverted repeats and can't distinguish between *T. brucei* spp. and *T. evansi*.

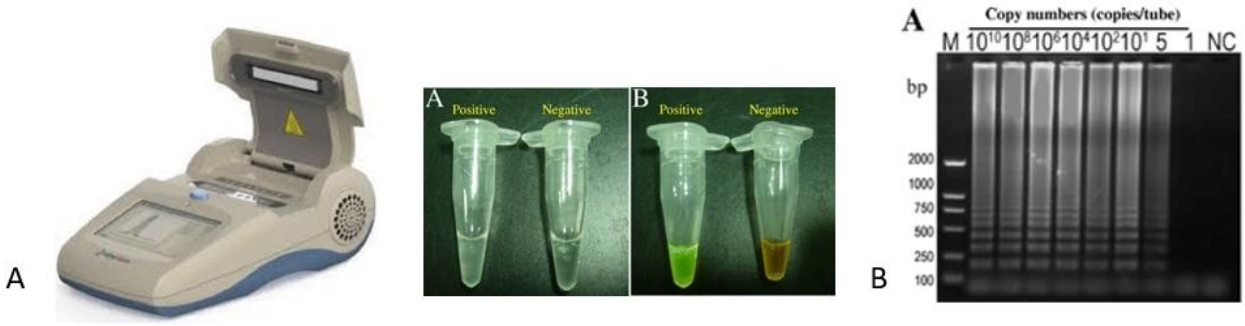

Figure 2.8: Visual illustration loop-mediated isothermal amplification (LAMP) (A), a gel visualizing LAMP product (B) (Zhang *et al*., 2010; Ogunjimi *et al*., 1999).

2.1.5.7. (b) Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an enzyme driven, molecular technique invented by Kary Mullis in 1983 which rapidly synthesize thousands to millions of a single or few copies of a target DNA fragment from a complex mixture of DNA for diagnostic and experimental purposes (figure 2.9) (Sherwoord *et al.,* 2014). PCR is used in medicine, biotechnology and microbial biology and has also become an essential part of certain diagnostic tests (Atz *et al.*, 1999). It is also used in cloning technique and forensic sciences. PCR is closely related to the natural principle of DNA replication where it allows the amplification *in vitro* of a specific region of DNA where specific primers are used to bind to the DNA template in a media containing an excess of denucleotide triphosphates (dNTPs) (Atz *et al.*, 1999). The amount of amplified product is limited to the available substrates in the reaction. *Taq* polymerase, is a thermostable DNA polymerase originally isolated from the thermophilic bacterium *Thermus aquaticus*, is used to synthesize the complementary strand (Sherwoord *et al.,* 2014). It consists of repeated reactions called cycles and is a three-step process that are precisely executed in a thermocycler (Sherwoord *et al.,* 2014). A thermocycler is an instrument that automatically controls and changes the temperatures for the appropriate time and temperature of the PCR cycles (Atz *et al.*, 1999).

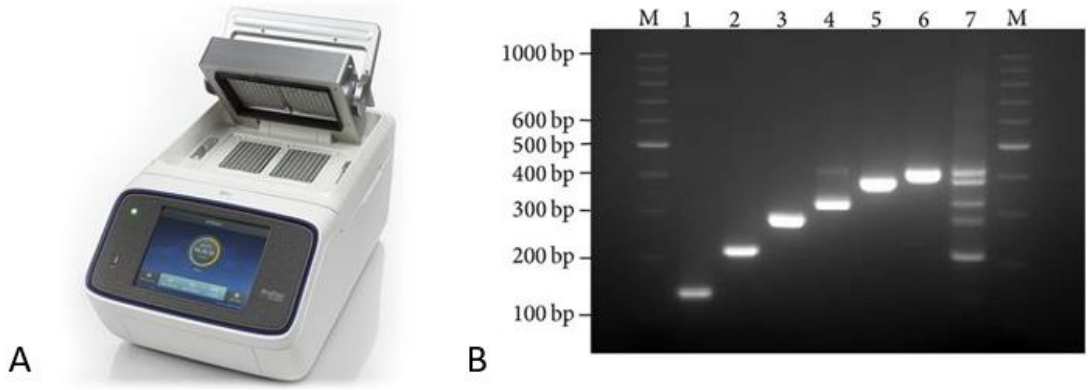

Figure 2.9: Visual illustration polymerase chain reaction (PCR) (A), a gel visualizing PCR product (B) (Wang *et al*., 2014; Applied Biosystems., 2019).

Conventional PCR is used to indicate and amplify the presence of a species, but the quantitative information isn't given (Hasson *et al*., 2012). Real-time PCR or qPCR is a method that also gives the quantitative information. It can determine the amount of DNA/RNA by adding fluorescently labelled probes to the reaction mixture and using specific thermocyclers it can be determined (Sherwood *et al*., 2014).

Masiga *et al*. (1996), developed oligonucleotide primers specific to *Trypanosoma simiae, T. congolense, T. brucei* and *T. vivax* species (Buscher *et al.*, 2014). PCR cannot be used for field diagnosis because it isn't cost effective and is a time-consuming assay. Trained professionals should perform the tests (Buscher *et al.*, 2014).

2.1.6. Treatment and control

For the treatment of Nagana or animal African trypanosomiasis three compounds can be used namely; isometamidium chrloride that has prophylactic properties, homidium bromide that is a therapeutic agent with limited prophylactic properties and diminazene aceturate that is also a therapeutic agent (Holmes *et al*., 2004). Aminoquinaldine derivate quinapyramine can also be used for animal trypanosomiasis according to Steverding (Steverding, 2008).

Surra caused by *T. evansi* can be treated by drugs such as isometamidium chloride, diminazene aceturate and homidium bromide (Hofkin & Loker, 2015).

Dourine caused by *T. equiperdum* can be treated by drugs such as suramin, diminazene, quinapyramine and cymelarson (Brun *et al.*, 1998). The same drugs used for treating Surra can be used for dourine as well (Brun *et al.*, 1998).

Treatment for sleeping sickness will be most effective when combined with chemotherapy that stops the multiplication of the parasite (Uilenberg, 1998). The first stage can be treated with pentamidine or suramin (Maudlin *et al.*, 2012). Stage two can be treated with melarsoprol although resistance has reduced the effectiveness of this drug (Maudlin *et al.*, 2012). Melarsoprol also has a 5% fatality rate because of the 'reactive arsenic encephalopathy' (Maudlin *et al.*, 2012). Eflornithine can also be used and diminazene aceturate is in the testing process for the possibility as medicine for sleeping sickness (Burri *et al*., 2004). New drugs are needed for the treatment of sleeping sickness (Maudlin *et al.*, 2012).

Measures for prevention focuses mostly on the control of tsetse flies and tabanid flies (Troncy *et al*., 1989). The use of chemical control can be implemented using insecticides or insect repellents. Biological control can be used by the means of the release of natural enemies that can reduce the fly populations. Breeding processes can be used to breed trypanotolerant cattle and are divided into two groups. The first group is the long-horned taurine group and the second group is the short-horned taurine group. Genetic control can also be used by means of the sterile insect technique (Troncy *et al*., 1989). Cultural control strategies can be used by the proper disposal of manure and waste near animals for the reduction of larval development (Hall & Wall, 2004).

For *T. equiperdum* castration as well as controlled mating can prevent spreading of the disease. Screening of animals before introducing the animals into a herd is necessary (Claes *et al*., 2005).

2.2. Toxoplamosis

Toxoplasma gondii is a large, obligate intercellular protozoan parasite with only one species known as *T. gondii* (Dubey & Petersen, 2001). *T. gondii* is distributed worldwide, with larger distribution in tropical areas, where cats serve as the definite host and warm-blooded animals as the intermediate host. The *T. gondii* infections are prevalent in humans and animals causing toxoplasmosis and human infections can become serious during pregnancy but infection is usually asymptomatic in healthy humans where the immune system prevent illness. It consists of three different stages (tachyzoites, bradyzoites and oocysts) that can be transferred to animals or humans by ingesting contaminated food or water or can be transferred congenitally.

Potential risks of toxoplasmosis are miscarriages in humans and animals, stillborn births or deformation of the foetus (Petersen, 2007). Other symptoms include enlarged lymph nodes, pulmonary necrosis, myocarditis (Sherwood *et al*., 2014). Congenital toxoplasmosis can cause mental disabilities, eye problems and disorders affecting the central nervous system (Urquhart *et al*., 1987). It can also be harmful to people or animals with a compromised immune system that can result in encephalitis, seizures, confusion, blurred eye vision or eye problems (Petersen, 2007).

2.2.1. Classification of *Toxoplasma gondii*

The classification of *Toxoplasma* according to (Dubey, 2010) is as follows:

Kingdom: Protista

Phylum: Apicomplexa

Class: Sporozoasida

Order: Eimeriorina

Family: Toxoplasmatide

Genus: *Toxoplasma*

Species: *Toxoplasma gondii*

2.2.2. Life cycle of *Toxoplasma gondii*

The life cycle is complex as seen in figure 2.10 (Hill & Dubey, 2002). It requires a definitive and intermediate host to complete the life cycle (Dubey, 2007). Sexual reproduction of the parasite will take place in the definite host whereas asexual reproduction will take place in the intermediate host (Gilot-Farmont *et al.,* 2012). Felids serve as the definite host by getting infected by either ingesting prey containing tachyzoites or bradyzoites or through direct transmission. The tachyzoites or bradyzoites will invade the intestinal epithelial cells in the definite host, multiply and excrete oocysts in faeces into the environment (Dubey, 2007). Sporulation will take place and form sporozoites that can remain latent for a long period of time and can be dispersed through water, soil and micro-fauna (Gilot-Farmont *et al*., 2012). The intermediate host will get infected by ingesting sporulated oocysts that release sporozoites which penetrate intestinal epithelium cells and other cells (Dubey, 2007). Endogeny will occur and form new tachyzoites that are released and will infect other surrounding cells. In the intermediate host the tachyzoites can move to any organ making it

possible to be transmitted through sexual transmission, milk, the placenta and through organ transplants and blood transfusions (Gilot-Farmont *et al*., 2012).

The immune system will respond to the infection by releasing antibodies which will limit the invasion of tachyzoites. This will result in the formation of cysts containing organisms, called bradyzoites, and are latent. Intermediate hosts can also get infected by the ingested of another intermediate host that contains bradyzoites or tachyzoites in the flesh (Urquhart *et al*., 1987). The life cycle will be repeated when the immune system wanes and infection will active again.

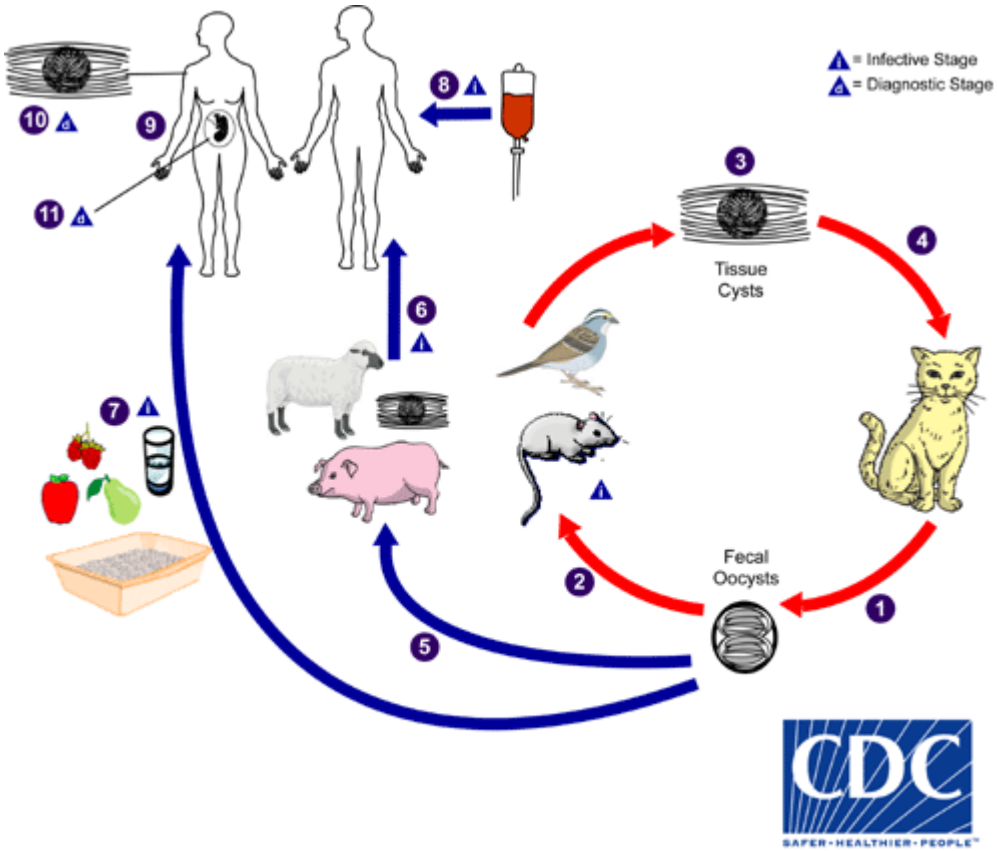

Figure 2.10. The life cycle of *Toxoplasma gondii* (Global Health, Division of Parasitic Diseases, 2018).

2.2.3. Diagnostics

The *T. gondii* infections can be diagnosed by a variety of ways including microscopic-, serological- and DNA-based diagnostic methods (Hill & Dubey, 2002). There is no available method to differentiate between oocysts and tissue cysts ingestion. Clinical signs are a

method not recommended for the diagnosis of *T. gondii* since the symptoms are very similar to a variety of other diseases.

2.2.3.1. Microscopic tests

Microscopic examination can be done for quick diagnostic analysis of samples (Hill & Dubey, 2002). Diagnosis using a light microscope is unreliable and gives untrustworthy results, but an electron microscope can be used (figure 2.3) (Liu *et al*., 2015). Tissue cysts can be distinguished by staining the cysts. Common staining techniques used include Giemsa, haematoxylin and eosin staining (figure 2.11 (A)). Periodic acid Schiff staining can also be used to stain bradyzoites (figure 2.11 (B)) (Hill & Dubey, 2002). To be able to perform these techniques skills and time is required and these techniques are cost effective (Liu *et al*., 2015).

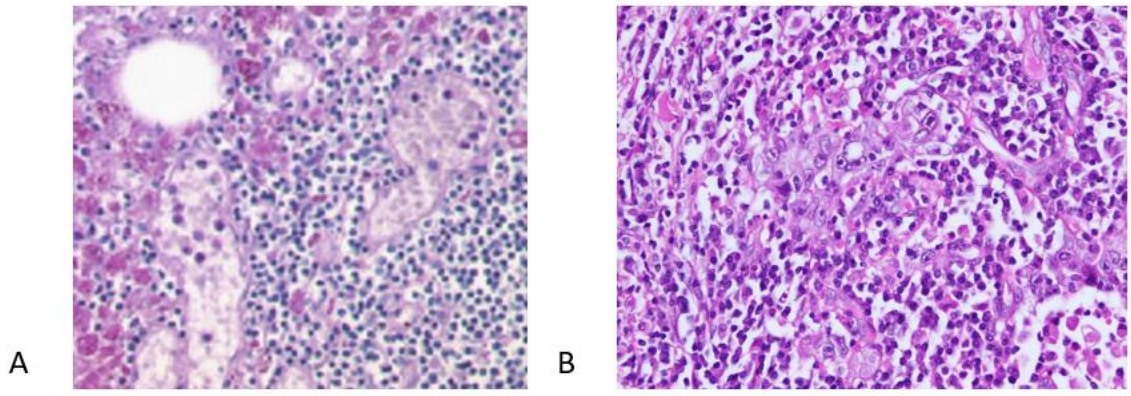

Figure 2.11: Visual illustration of periodic acid Schiff staining (A) and haematoxylin and eosin staining (B) (Moos & Schneider, 2011, Porcaro *et al*., 2003).

2.2.3.2. Isolation test

A bioassay can be performed isolating *T. gondii* using laboratory animals or any human tissue or body fluid (Montoya, 2002). Unfortunately, skills and time is required and is very costly (Liu *et al*., 2015).

2.2.3.3. Serological tests

Numerous serological tests can be used for the detection of antibodies and antigens (Hill & Dubey, 2002). These tests include “the Sabin–Feldman dye test (DT), the indirect

hemagglutination assay (IHA), the indirect fluorescent antibody assay (IFA), the direct agglutination test, the latex agglutination test (LAT), the enzyme-linked immunosorbent assay (ELISA), and the immunosorbent agglutination assay test (IAAT)". Because immunoglobulin M (IgM) antibodies appear faster after infection than IgG antibodies some assays like the ELISA, IFA and IAAT has been modified to detect IgM antibodies (Hill & Dubey 2002). For whole parasites the Sabin–Feldman dye test (DT), the direct agglutination test and the indirect fluorescent antibody assay (IFA) (Hennawy, 2016). For disrupted parasites test that can be used for detection include ELISA, latex agglutination test (LAT), indirect hemagglutination assay (IHA) and compliment fixation. IgM antibodies is found to have a low sensitivity and can give false-negatives it is necessary to first test for IgG antibodies and if positives are found then test for IgM for acute infections (Hennaway, 2016).

2.2.3.4. Sabin-Feldman Dye Test (DT)

Developed by Sabin and Feldman in 1948, this test is still used to detect anti-*T. gondii* antibodies. Although the DT is used, results indicate that the DT is more sensitive and specific for human samples than animal samples (Dubey, 2014). Other disadvantages include skill is required to perform the DT (Ramirez *et al*., 2017). Because live parasites are needed for the DT and that means that specialized technology is needed for maintaining the parasites. It also possesses a risk to technicians performing the test (Ramirez *et al*., 2017).

2.2.3.5. Agglutination tests

Tests included are modified agglutination tests (MAT), Latex agglutination test (LAT), Indirect hemagglutination test (IHA) and the Immunosorbent agglutination test (ISAGA) (Liu *et al*., 2015) (figure 2.12).

MAT is used to detect IgG antibodies and is quite sensitive and specific if the correct preservative is used to prepare the antigen. A positive sample will result in a thin mat of agglutination. It is also the agglutination test that is used most commonly (Montoya, 2002). LAT is used to detect anti-*T. gondii* IgG antibodies and has also been modified for detecting anti-*T. gondii* IgM antibodies in humans. LAT is more sensitive and specific in human samples than animal samples. IHA detection is the same as LAT and a positive sample indicates agglutination. IHA takes longer to show the result of a positive sample and thus not recommended to be used in acute and congenital infections. ISAGA is a test that is seen to be easier done than ELISA, but it requires large numbers of tachyzoites. ISAGA is

performed on a plate where IgM antibodies bind to the plate coated in anti-species IgM and agglutination will occur giving a positive result (Liu *et al*., 2015).

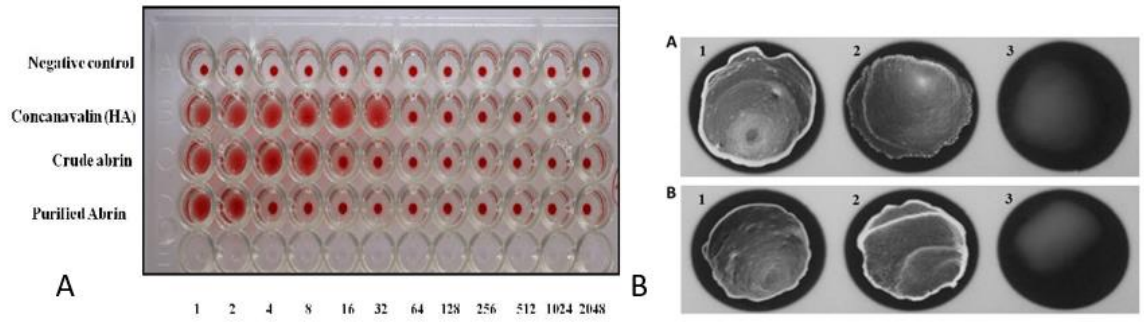

Figure 2.12: Visual illustration of hemagglutination tests MAT, IHA and ISAGA (A), and latex agglutination test (LAT) (B) (Chaturvedi, *et al*, 2015, Rezaei *et al*., 2019).

2.2.3.6. Indirect fluorescent antibody test (IFAT)

IFA is used for the detection of antibodies for the detection of *Toxoplasma gondii* (Dubey, 2014). The assay causes an antibody-antigen reaction and detection can be seen by fluorescence using a fluorescence microscope (Ramirez *et al*., 2017). It possesses the potential for high precision when adapted for specific antibodies. Disadvantages include a fluorescence microscope is needed and results given by the individual performing the test, making variation from individuals are possible (Liu *et al*., 2015).

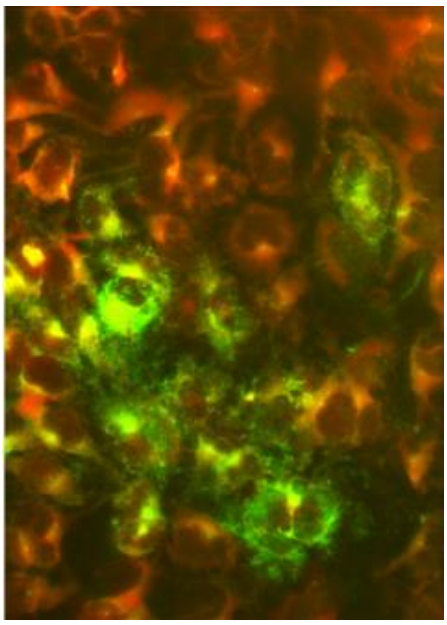

Figure 2.13: Visual illustration of indirect fluorescent antibody test (IFAT) (Rudd, *et al.,* 2013).

2.2.3.7. Enzyme-linked immunosorbent assay (ELISA)

As explained previously the method works the same as for *Trypanosoma spp.* but detection is for antibodies and antigens for *T. gondii* (Ramirez *et al*., 2017)

Other serological tests that can be used is the immunochromatographic test (ICT), Western blotting (WB), Piezoelectric immunoagglutination assay (PIA), avidity test and imaging techniques (Liu *et al*., 2015).

2.2.3.8. DNA-based diagnostic methods

Similar molecular diagnostic methods used for *Trypanosoma spp*. can also be used for *T. gondii*. These include conventional PCR, Real-time PCR, LAMP, restriction enzyme fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (ALFP), multilocus sequence typing (MLST) (figure 2.8 and figure 2.9).

Target genes that are used for *T. gondii* when performing PCR includes the B1 gene, 529 bp repeat element, 18S rDNA or internal transcribed spacer (ITS-1) (Liu *et al*., 2015). Specificity is very dependent on the DNA extraction method used (Ramirez *et al*., 2017). Real-time PCR is the best performing technique with LAMP being slightly less sensitive. LAMP have been found to produce several false positives and sequencing is expensive (Suleman *et al*., 2016). Real-time PCR and nested PCR uses the B1 gene as target gene for diagnosis (Liu *et al*., 2015).

2.2.4. Treatment and control

Sulphonamides or a mix between sulphonamides or sulphadiazene and pyrimethamine have been found to be effective against tachyzoites (Hill & Dubey, 2002). The activity of sulphonamides and pyrimethamine is improved using folic acid and yeast (Dubey, 2010). Reports indicated that spiramycin has anti-toxoplasmic activities. Clindamycin can be used to reduce oocyst shedding in cats and have anti-toxoplasmic activity but won't eliminate the oocysts completely. (Urquhart *et al*., 1987).

To prevent and control the parasite it is important to screen pregnant woman and they must stay clear of any cat litter boxes, raw meat and soil (Hill & Dubey, 2002). Buy commercial food to feed cats, litter boxes must be cleaned regularly, and the waste must be properly disposed of. Hygiene measures can be taken by wearing gloves when gardening and cleaning of food and hands when working with food. Meat can be introduced to extreme heat

or cold to kill the parasite in the meat and do not eat raw meat (Hill & Dubey, 2002). A vaccine is available to reduce fetal losses in sheep namely TS-4 vaccine and T-263 vaccine/ T-263 vaccine is used to prevent cats from shedding oocysts (Dubey, 2010).

2.3. Cryptosporidiosis

Cryptosporidium is a genus of obligate protozoan parasite containing a variety of species where some causes waterborne diseases called cryptosporidiosis (Sattar & Springthorpe, 1999). These protozoan parasites can invade the epithelial cells of the intestines, respiratory tract or the gastrointestinal tract of mammals, reptiles, birds and fish that can be found worldwide (Roberts *et al*., 2013). Studies indicate that *Cryptosporidium* is more closely related to gregarines than coccidians explaining the resistance to anti-coccidial drugs. These species sizes range from 2 µm – 6 µm where oocysts can only be seen in faeces. The oocysts contain no sporocysts but contains four sporozoites that will invade the epithelial cells when the oocysts get swollen. What makes this genus so interesting is the fact that sporulation takes place within the host (Loop *et al*., 1998). Hosts that *Cryptosporidium* species can infect include farm animals, wildlife, dogs and cats, reptiles, fish, birds and different rodents. Some species are more host specific than other (Gunn & Pitt, 2012).

It is known to cause cryptosporidiosis in humans and animals that is a diarrhoeal disease (Gunn & Pitt, 2012). Cryptosporidiosis can be distributed through contaminated water, infected food sources contaminated with faeces containing oocysts. The oocysts are very small meaning that it is often difficult to remove from water through sand filtration systems and can be viable up to 6 months in moist conditions (Sherwood *et al*., 2014). The oocysts can also build resistance against disinfectants like chlorine (Sherwood *et al*., 2014). Cryptosporidiosis is seen as a causative agent of chronic diarrhoea and is regarded as a significant problem (Roberts *et al*., 2013). The predominant cause of cryptosporidiosis is *C. parvum*. These protozoan parasites can invade the gastrointestinal tract of all vertebrates and can be found worldwide (Fayer, 2008). Neonatal animals, animals and humans that are immuno-comprised or malnourished are especially at risk. Symptoms can vary in degree from subclinical to severe because different factors play a role like immune and nutrition (Gunn & Pitt, 2012). Symptoms include abdominal cramps, fever and severe diarrhoea, dehydration, weight loss, nausea, fatigue and sometimes swollen lymph nodes (Fayer *et al*., 2017). When infection occurs in the respiratory tract symptoms also include coughing, excess mucus, sneezing and respiratory distress.

2.3.1. Classification of *Cryptosporidium* parasites (Fayer *et al*., 2017)

Kingdom: Protista

Phylum: Apicomplexa

Class: Sporozoasida

Order: Eucoccidiorida

Family: Cryptosporidiidae

Genus: *Cryptosporidium*

Species: *Cryptosporidium parvum*

Cryptosporidium hominis

Cryptosporidium meleagridis

Cryptosporidium erinacei

2.3.2. Life cycle of *Cryptosporidium*

The life cycle of *Cryptosporidium spp*. is described and illustrated in figure 2.14 (Bouzid *et al*., 2013). A host gets infected by encountering contaminated food, water or oocysts in the general environment (Fayer *et al*., 2017). Excystation take place when the respiratory system or small intestine is reached by ingested oocyst. Then followed by the release of four sporozoites attaching to the epithelial cells. Infection can be increased through factors such as the presence of bile salts, pH and temperature and cause the infection to spread to other tissues. Chemicals are discharged from the sporozoites forming protrusions that result in encapsulation within a parasitophorous vacuole (Tzipori & Widmer, 2000). The vacuole expands to surround both the parasite and host cell components being separated from the host cytoplasm making it extracytoplasmic. Next the apical membrane of the host will fuse with the vacuole, known as the annular ring. Another organelle develops, known as the feeder organelle, taking up nutrients from the host cells. The sporozoites will differentiate to trophozoites (Fayer *et al*., 2017).

Schizogony (asexual reproduction) will take place developing in two successive generations of merogony (Tzipori & Widmer, 2000). The first is type one meronts releasing eight merozoites where type two meronts releases four merozoites (Gunn & Pitt, 2012). Type two meronts are responsible for gametogony (sexual reproduction) producing macrogamonts and microgamonts. Fertilization will follow next forming zygotes. Zygotes develop into thin-walled and thick-walled oocysts. The thin-walled oocysts are known to re-infect the host

while the thick-walled oocysts are released into the environment as oocysts by either faeces or nasal/ respiratory secretion that has the potential to infect another host (Fayer *et al*., 2017).

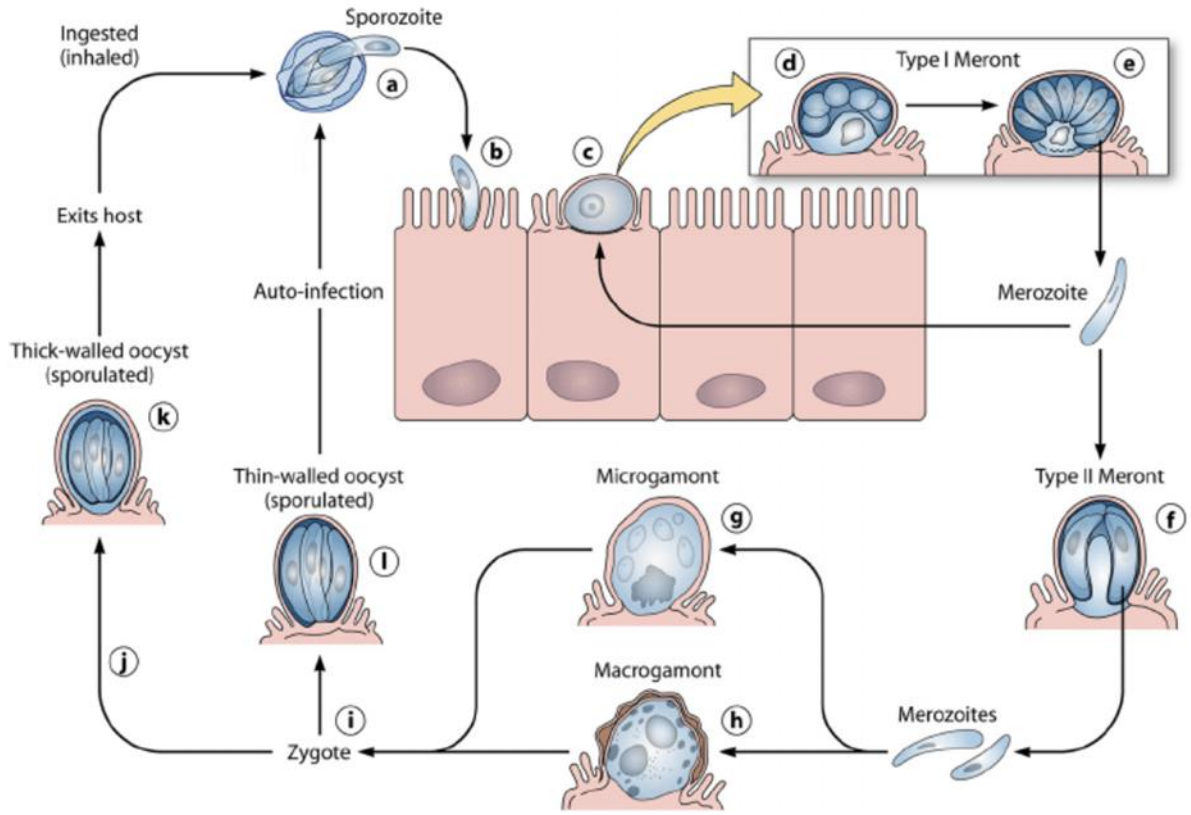

Figure 2.14: The life cycle of *Cryptosporidium spp*. (Bouzid *et al*., 2013).

2.3.3. Diagnostics

Different types of techniques can be used to detect *Cryptosporidium* spp. infections including microscopic-, serological- and molecular tests.

2.3.3.1. Microscopic tests

Wet mount examination can be used but unstained samples through a light microscope is usually missed (figure 2.3) (Khurana & Chaudhary, 2018). It isn't very sensitive but can be used to detect oocysts in faecal samples (Bouzid *et al*., 2013). Most species also look very identical under a microscope (Xiao *et al*., 2015). The diagnosis cannot exclude Cryptosporidium either because the sample can be below detection numbers needed for

diagnosis (Chalmers & Davies, 2010). Electron microscopy can be used but is complex and expensive (Khurana & Chaudhary, 2018).

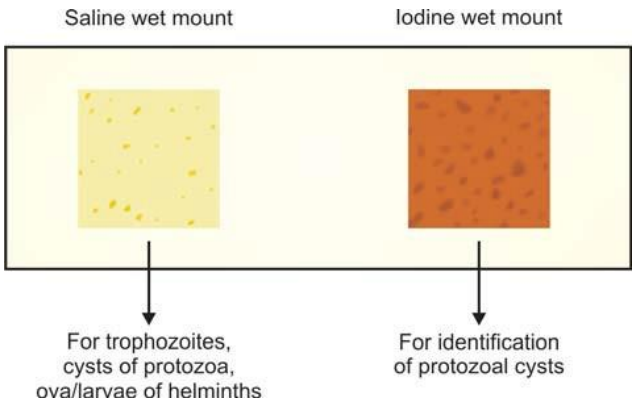

Figure 2.15: Visual illustration wet mount examination (BS media, 2017).

Methods to increase the concentration of oocysts and to remove debris from the sample are include Sheather's sucrose flotation method, centrifugation at a high speed, saturated salt flotation method and Allen and Ridley's formal-ether method (Khurana & Chaudhary, 2018). Indirect immunofluorescent microscopy can be used to identify oocysts in environmental samples. All these methods are time consuming and needs to perform with skill.

A biopsy of the intestinal mucosa tissue can be extracted for diagnosis but isn't recommended because not all regions of the intestine may be infected, and the sample requires delicate processing.

2.3.3.2. Staining methods

Giemsa and Jenner's stain have been used for the identification of oocysts in faecal samples, but this test is insensitive and non-specific (figure 2.3). A modified version of acid-fast Ziehl-Neelsen stain and Kinyoun's acid-fast stain can be used to identify oocysts but the results can be confusing due to staining of other various structures. Other staining methods include Safranin-methylene blue stain and fluorogenic stain auramine-phenol (figure 2.13) (Khurana & Chaudhary, 2018).

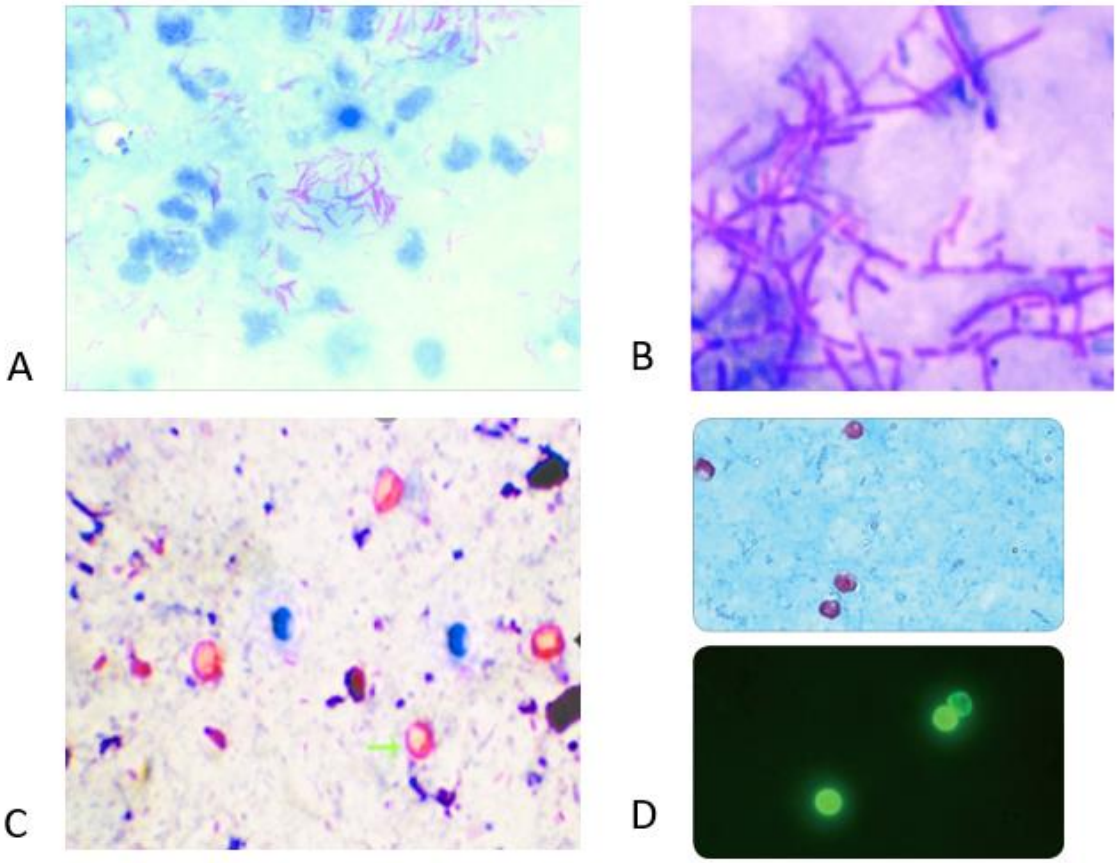

Figure 2.16: Visual illustration of acid-fast Ziehl-Neelsen stain (A) and Kinyoun's acid-fast stain (B), Safranin-methylene blue stain (C), fluorogenic stain auramine-phenol (D) (Prasad *et al*., 2011; Branco *et al*., 2015; Zintl *et al.,* 2006; Rehka *et al*., 2016)

Direct immunofluorescence assay (DFA) has a higher sensitivity and specificity rate than acid-fast staining (figure 2.14). Using an immunofluorescence microscope, oocysts can be detected but species cannot be identified from each other (Xiao *et al*., 2015).

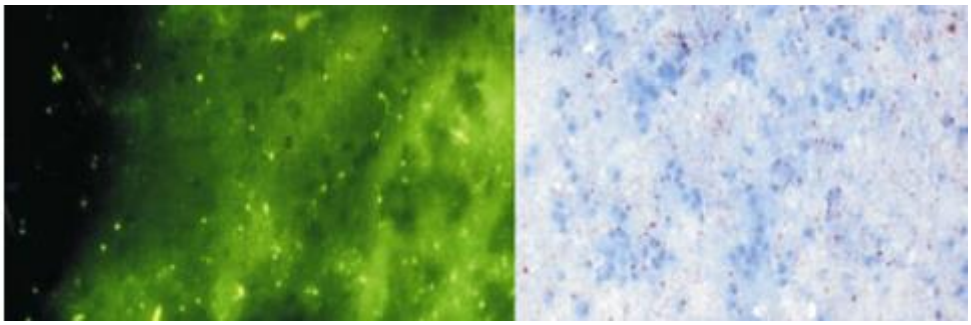

Figure 2.17: Visual illustration of direct immunofluorescence assay (DFA) (Madhusudana *et al*., 2012).

2.3.3.3. Serological tests

Antigen-capture-based enzyme immunoassay (EIA) is used regularly in human samples but in animal samples results can be comprised since this depends on how specific the antibodies are (Xiao *et al*., 2015). Enzyme-linked immunosorbent assay (ELISA) can be used depending on which antibody is used, the percentage of the sensitivity will be influenced. Specificity on the other hand will be high (Savioli *et al*., 2006). ELISA can facilitate testing of a large number of samples (figure 2.7) (Chalmers & Davies, 2010). Immunochromatographic test (ICT) can be used with stool samples for detection, but ICT gives high false-positives (Chalmers & Davies, 2010). Antibody detection methods are mostly not used because it reflects on either a past or current infection and is rather used for sero-epidemiological surveys of the disease (Khurana & Chaudhary, 2018).

2.3.3.4. DNA based tests

Various methods can be used including conventional PCR, RFLPs, real-time PCR, LAMP, multiplex allele-specific-PCR (MAS-PCR) and nested PCR (figure 2.8 and figure 2.9) (Khurana & Chaudhary, 2018). Different target genes can be used for these molecular tests. These target genes are 18S rRNA, TRAP C1, COWP, HSP70, DHF and GP60 genes. All these methods are time consuming and labour intensive (Xiao *et al*., 2015).

2.3.4. Treatment and control

In some animals and humans, it is mostly seen as a self-limiting disease where the host is usually healthy (Olson *et al*., 2003). In farm animals' drugs that can be administered include paramomycin, halofuginone lactate and lasalocid. Some of the drugs are sometimes too expensive or toxic when used effectively. An alternative is the use of fluid therapy and the modification of acid base disturbances. Colostrum can be used to reduce oocyst release, diarrhoea and secondary infections. Oral treatment of monoclonal antibodies can also be applied (Roberts *et al*., 2013). Nitazoxanide combined with rehydration can be used as treatment (Sherwood *et al*., 2014). To prevent further spread of an infection after a breakout with farm animals' disinfection of buildings is necessary usually with ammonia-based disinfectants or steam cleaning (Loop *et al*., 1998). Separation of infected and uninfected animals is a mechanical mechanism that is also necessary.

2.4. High Resolution Melt quantitative real-time PCR (HRM-qPCR)

HRM-qPCR is the combination of qPCR and HRM to form a method that is suitable to test a large series of samples that is highly sequence specific and sensitive (Rouleau *et al.*, 2009). HRM analysis was developed because qPCR did not possess the capabilities to distinguish small differences between melting curves (figure 2.15) (Vossen *et al*., 2009). HRM-qPCR analysis is a great method that is based on PCR melting curve techniques that can discriminate DNA sequences by the composition length, GC-content or melting temperatures and is sensitive enough to discriminate between single nucleotide differences. It is an upcoming technique that is very cost effective, rapid and simple making it ideal to use for diagnostics (Rouleau *et al.*, 2009). Primers gets specifically designed for the HRM-qPCR test by obtaining a sequence of a targeted gene. After obtaining this information primer design software can be used to finish designing the primers to identify different species (Pestana *et al*., 2010).

The difference between standard melt curves and HRM analysis is that brighter fluorescent dye is used at higher concentrations (Life Technologies Corporation, 2010). It has the ability to “collect fluorescence data at finer temperature resolutions” and uses new algorithms and plots with new refined software. The method includes a q-PCR stage where amplification occurs in the target region with fluorescent dye. When the reaction begins the presence of double stranded DNA is high while the temperature is low and the fluorescence dye strong. As the temperature increases the fluorescence dye decrease and the double stranded DNA gets denatured to single stranded DNA (Life Technologies Corporation, 2010). The second stage include a high-resolution melt analysis capturing fluorescent data per change in the temperatures. Fluorescence is used that gets emitted and creates a unique curve dependant on the designed primers.

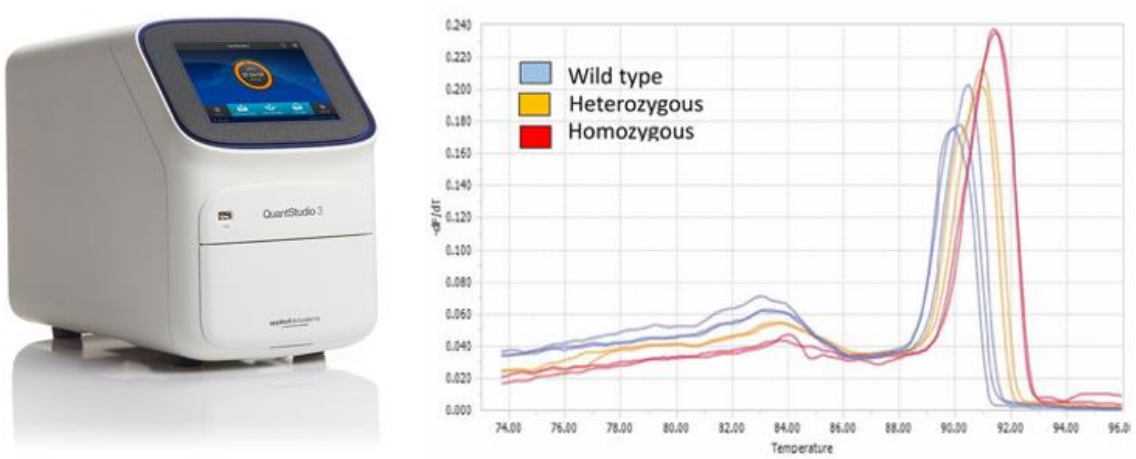

Figure 2.18: Visual illustration of a Quantstudio 3 qPCR machine and results of HRM-qPCR analysis (Onasanya *et al*., 2018; Thermo Fisher Scientific, 2019).

HRM-qPCR us a new and upcoming technique for diagnostics and that is why it was chosen for this study since it has not been developed for *Trypanosoma* spp., *Cryptosporidium* and *T. gondii* and is a new method for these parasites.

CHAPTER 3

MATERIALS AND METHODS

3.1. Target gene identification

Target gene regions were identified from published articles of PCR assays for the detection of *Trypanozoon* spp., (Njiru *et al*., 2008), *T. congolense* (Kuboki *et al*., 2003), *Cryptosporidium* spp. (Morgan *et al*., 2001) and *Toxoplasma* (Cardona *et al*., 2011) as seen in tabel 3.1.

Tabel 3.1: Target gene identification for different genera

Genus	**Target gene**	**References**
Trypanozoon spp.	RIME gene	Njiru *et al*. (2008)
T. congolense	rP0 gene	Kuboki *et al*. (2003)
Cryptosporidium spp.	HSP70 gene	Morgan *et al*. (2001)
Toxoplasma gondii	B1 gene	Cardona *et al*. (2011)

3.1.1. *Trypanosoma*

3.1.1.(a) Subgenus *Trypanozoon*

For subgenus *Trypanozoon* spp. different target genes were identified for use in this study including the repetitive insertion mobile element (RIME), serum resistance associated (SRA) gene and *T. b. gambiense*-specific glycoprotein (TgsGP) gene. The SRA gene is mostly used for the identification of *T. b. rhodesiense* due to the presence of serum-resistant variants while being absent in *T. b. brucei* and *T. b. gambiense* (De Greef *et al*., 1992; Njiru *et al*., 2004). TgsGP gene is mostly used for the detection *T. b. gambiense* due to the expression of a 47 kDa receptor-like glycoprotein in *T. b. gambiense (*Gibson *et al*., 2010*)*. The RIME gene is found to be specific to the subgenus *Trypanozoon* species (Njiru *et al*., 2008).

3.1.1.(b) *Trypanosoma congolense*

The target gene identified for *T. congolense* was ribosomal protein P0 (rP0) gene. The *T. congolense* primers for HRM-qPCR were designed from the ribosomal P0 gene which is specifically found in *T. congolense* (Kuboki *et al*., 2003).

3.1.2. *Cryptosporidium*

It was found that different target gene regions used for *Cryptosporidium* included 60-kDa glycoprotein (GP60), *Cryptosporidium* glycoprotein gp40/15 (gp40/15) and 70-kDa heat shock protein (HSP70). Published articles indicated that gp40/15 gene were frequently used for distinguishing between *C. parvum* and *C. hominis* (Wanyiri *et al*., 2007). The GP60 gene was also found to distinguish between *C. parvum* and *C. hominis* while GenBank mostly identified *C. parvum* sequence data (Stensvold *et al*., 2014). The target gene HSP70 was chosen due to the high diversity of *Crytosporidium* species that can be identified and distinguished from one another. HSP70 gene was further used in this study since recent studies shown that it is seen as a multi-species target gene and can identify and distinguish between several *Cryptosporidium* species (Sulaiman *et al*., 2000). Available sequence data on NCBI GenBank also support the variety of *Cryptosporidium* species that were included.

3.1.3. *Toxoplasma*

Previously published primers targeting the B1 gene which is specific to *T. gondii* were used for HRM-qPCR (Zhou *et al*., 2018).

3.2. High Resolution Melt qPCR primer design

The primers were designed by using the recommendations of Wojdacz & Lotte Hansen (2006) and Smith *et al*. (2009) to prevent PCR bias. Firstly, CpG sites must be kept at a minimum and should occur as close to the 5’ end of the primer sequence as possible (Wojdacz & Lotte Hansen, 2006). The annealing temperature should be optimized to improve the sensitivity of the assay due to the fact at low annealing temperatures unmethylated templates will amplify more while at increased annealing temperatures methylated templates are amplified, reducing PCR bias (Wojdacz & Lotte Hansen, 2006). The primer length should be between 20 bp-30 bp and the melting temperature of the primer pair should be similar (Smith *et al.*, 2009). There should be a limited complimentary

sequence in primers and between primer pairs to prevent primer-dimers. This was confirmed using Vector NTi software. The amplified regions must contain conserved regions for genus identification and differential regions to distinguish between species (Smith *et al.,* 2009).

3.2.1. Subgenus *Trypanozoon* species

The subgenus *Trypanozoon* species primers for HRM-qPCR were designed from the RIME gene which is specifically found in *T. b. brucei, T. b. rhodesiense, T. b. gambiense, T. evansi* and *T. equiperdum* (Njiru *et al*., 2008). Additional *Trypanosoma* RIME sequences were downloaded from the NCBI database: *T. b. rhodesiense* (accession number: EF567424), *T. brucei brucei* (accession number: EF567426), *T. evansi* (accession number: EF567425). The target gene regions were used to download sequences from the NCBI Genbank database and used in Vector NTi software for sequence alignment to be performed. The names were edited to represent the *Trypanozoon* species and for distinguishing purposes. Primers were then chosen and designed from the sequence alignment to target regions of significant variation between *Trypanozoon* species.

3.2.2 *Trypanosoma congolense*

Additional *T. congolense* rP0 sequences were downloaded from the NCBI database: *T. congolense* isolates (accession number: AB056702). The target gene regions were used to download sequences from the NCBI GenBank database and used in the software program Vector NTi for sequence alignment to be performed. The names were edited to represent the *T. congolense* species and for distinguishing purposes. Primers were then chosen and designed from the sequence alignment to target regions of variation.

3.2.3. *Cryptosporidium* species

Cryptosporidium species primers were designed from the HSP70 gene which is specifically found in genus *Cryptosporidium* (Morgan *et al*., 2001). Additional *Cryptosporidium* HSP70 sequences were collected from the NCBI database: *C. parvum* isolates (accession numbers: AF221530, DQ898162), *C. hominis* isolates (accession numbers: KR296809, KM116516, KM116517, KU892576, KU892578), *C. meleagridis* isolates (accession numbers: AF402284, AF329189) and *C. erinacei* isolates (accession number: KF612325). The target gene regions were used to download sequences from the NCBI Genbank database and used in the software program Vector NTi for sequence alignment to be performed. The names were

edited to represent the *Cryptosporidium* species and for distinguishing purposes. Primers were then chosen and designed from the sequence alignment to target regions of variation.

3.2.4. *Toxoplasma gondii*

T. gondii species primers were designed from the B1 gene which is specific to *T. gondii* (accession number: AF179871). The target gene regions were used to download sequences from the NCBI Genbank database and used in the software program Vector NTi for sequence alignment to be performed. The names were edited to represent *T. gondii* species for recognition purposes. Primers were then chosen and designed from the sequence alignment to target regions of variation.

3.3. g-Block design

g-Blocks (synthetic target DNA) were designed by using the sequences from the NCBI Genbank database for each of *Trypanozoon* species RIME gene, *T. congolense* rP0 gene, *Cryptosporidium* species HSP70 gene and *T. gondii* B1 gene and the primers designed from the sequences to identify target regions for gBlock design. The g-Blocks were used as positive control DNAs to determine the sensitivity and specificity of the assay for assay optimization. A series of dilutions of different gBlock DNA concentrations were used during the reactions and included several species in the analysis. uMelt curve software was used to see the predicted melting curves of each gBlock sequence that were designed and ordered from Thermo Fisher Scientific (Dwight *et al*., 2011).

3.4. Optimization for High Resolution Melt-qPCR with HRM primers

3.4.1 Annealing temperature and target gene optimization

HRM-qPCR was performed for the optimization of the annealing temperature and specificity using the g-Blocks and primers designed for *Trypanozoon* spp*, T. congolense*, *Cryptosporidium* spp. and *T. gondii*. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of g-Block DNA template (Dilutions 10^{-1} to 10^{-4} with 10^{-1} ~1 ng/µl). All reactions were performed in duplicate including negative controls. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. It was followed by 95°C for 15 seconds, then 60°C for 1 minute. A melt curve was constructed from 60°C to 95°C with

increments of 0.5/s followed by a hybridization step from 95°C to 60°C. The annealing temperature was altered as needed for successful melt curve analysis.

3.4.2. Sensitivity

To determine the sensitivity and detection limit of the HRM-qPCR assays a series of gBlock dilutions (Dilutions 10^{-1} to 10^{-8} with 10^{-1} ~1 ng/µl). were tested using primers designed for *Trypanozoon* spp*, T. congolense*, *Cryptosporidium* spp. and *T. gondii*. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of g-Block DNA template (Dilutions 10^{-1} to 10^{-4} with 10^{-1} $\sim 1\ ng/\mu l$). All the reactions performed included a negative. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. It was followed by 95°C for 15 seconds, then 60°C for 1 minute, followed again by 95°C for 15 seconds and 60°C for 15 seconds. A melt curve was constructed from 60°C to 95°C with increments of 0.5/s followed by a hybridization step from 95°C to 60°C. The annealing temperature was altered as needed for successful melt curve analysis.

3.4.3. High resolution melt-qPCR analysis

HRM-qPCR was performed for the screening of the different protozoan parasites that include *Trypanozoon* spp., *T. congolense*, *Cryptosporidium* spp. and *T. gondii.*

3.4.3.1. Amplification of *Trypanozoon* spp. DNA

Primers designed specifically to identify *Trypanozoon* spp. targeting the RIME gene were designed. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of DNA template. The reaction that was performed included a negative and positive control. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. It was followed by 95°C for 15 seconds, then 60°C for 1 minute. A melt curve was constructed from 60°C to 95°C with increments of 0.5/s followed by a hybridization step from 95°C to 60°C.

3.4.3.2. Amplification of *T. congolense* DNA

Primers designed specifically to identify *T. congolense* targeting the rP0 gene were designed. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of DNA template. The reaction that was performed included a negative and positive control. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A melt curve was constructed from 60°C to 95°C with increments of 0.5/s followed by a hybridization step from 95°C to 60°C.

3.4.3.3. Amplification of *Cryptosporidium* spp. DNA

Primers designed specifically to identify *Cryptosporidium* spp. targeting the HSP70 gene were designed. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of DNA template. The reaction that was performed included a negative and positive control. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A melt curve was constructed from 60°C to 95°C with increments of 0.5/s followed by a hybridization step from 95°C to 60°C.

3.4.3.4. Amplification of *T. gondii* DNA

Primers designed specifically to identify *T. gondii* targeting the B1 gene were designed. The reaction composed of a final volume of 20 µl which included 10 µl MeltDoctor HRM Mastermix (ThermoFischer Scientific, US), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 8.2 µl of double distilled water and 1 µl of DNA template. The reaction that was performed included a negative and positive control. Using the Quantstudio 3 (Thermo Fisher Scientific, US) the qPCR conditions were as follows: A initial hold at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A melt curve was constructed from 60°C to 95°C with increments of 0.5/s followed by a hybridization step from 95°C to 60°C.

3.5. Optimization for conventional PCR with HRM primers

3.5.1. Annealing temperature and target gene optimization

The different genus samples were tested at different annealing temperatures, X°C, starting with 60°C and adjusted accordingly for optimizing amplification of the target gene region. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template (Dilution 10^{-1} ~1 ng/µl). The reaction that was performed included a negative control of double distilled water and positive control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at X°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C ∞. PCR products were electrophoresed using 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

3.5.2. Detection limit

The different genus samples were tested for the detection limit using a series of gBlock dilutions (Dilutions 10^{-1} to 10^{-8} with 10^{-1} ~1 ng/µl) and primers designed for *Trypanozoon* spp*, T. congolense*, *Cryptosporidium* spp. and *T. gondii*. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template (Dilutions 10^{-1} to 10^{-8} with 10^{-1} ~1 ng/µl). The reaction that was performed included a negative control of double distilled water and positive control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at X°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C. PCR products were electrophoresed on a 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

3.6. Sample collection

A total of 20 sheep faecal samples were collected and pooled together to give 10 sheep faecal (n=10) samples from a communal farming area outside of Potchefstroom (26°73'35.65''S, 26°92'53.54''E) as seen in figure 3.1. Potchefstroom is a city that is in the North-West province of South Africa (Figure 3.2.). Seven Wild bird faecal (n=7) samples were collected at the North-West University (NWU) (26°69'05.18"S, 27°09'32.46"E) in Potchefstroom. The samples were transferred into collection vials and transported to the North-West University for analysis. Furthermore, archived (at NWU) DNA samples extracted from cattle blood and tsetse flies collected in previous studies from uMkhanyakude district of KwaZulu-Natal province (Figure 3.2) were also used for screening to test the HRM-qPCR and conventional PCR assay.

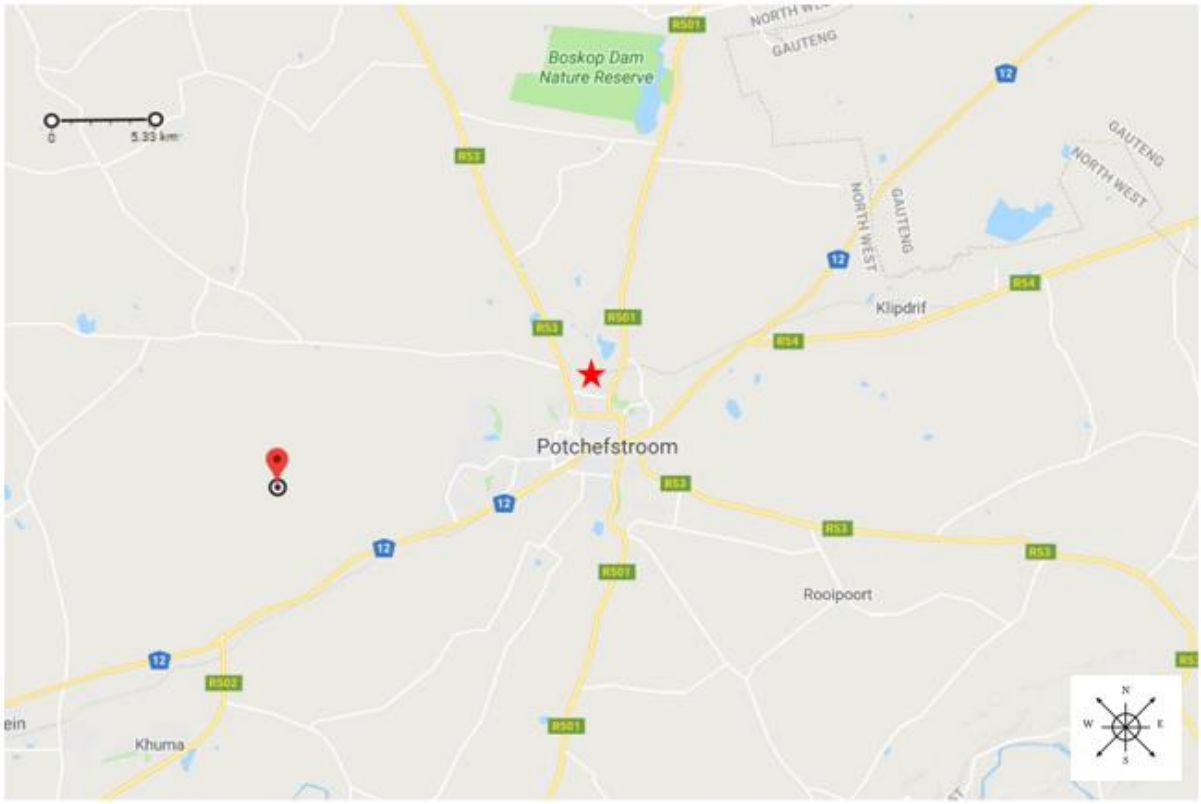

Figure 3.1: Location of Matlwang communal farming area (indicated by the asterisk) outside Potchefstroom where cattle and sheep faecal samples were collected.

Figure 3.2: Map of North West province (Wikipedia, 2019).

Additionally, archived DNA samples of cattle blood (n = 7) and tsetse flies (n = 10) that were previously collected from the uMkhanyakude district of KwaZulu-Natal province were also used for screening purposes.

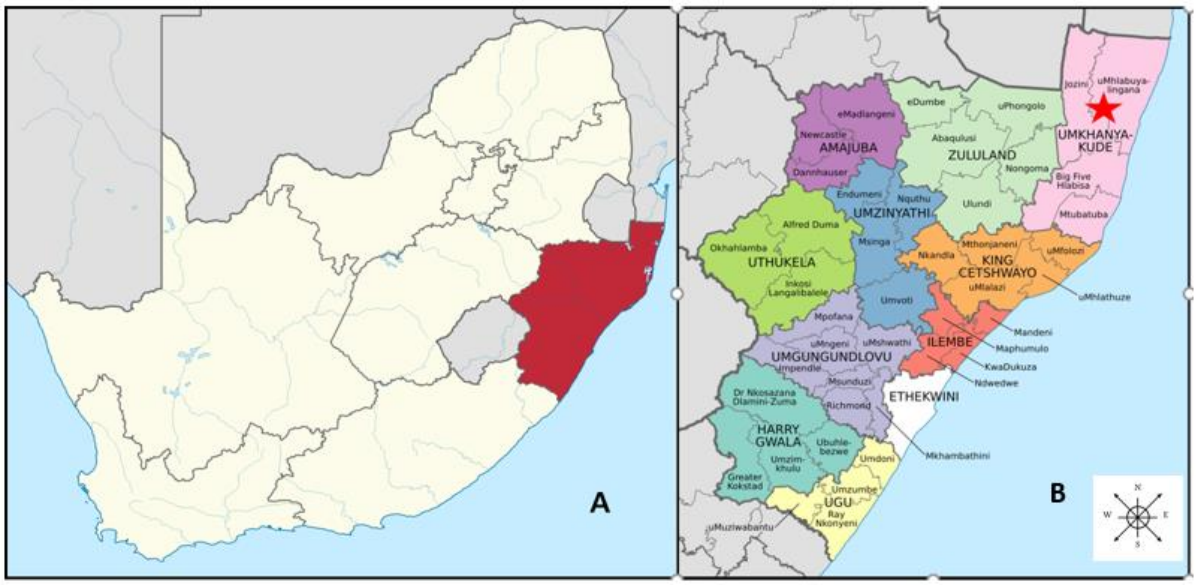

Figure 3.3: Map showing the sampled area. A) KwaZulu-Natal province. B) uMkhanyakude district (Wikipedia, 2018).

3.7. DNA extraction

The faecal samples were placed into 2 ml microcentrifuge tubes and placed into liquid nitrogen for 5 minutes and thawed. This step was repeated three times. A modified salting out method were used by Rivero *et al*. (2006). A volume of 480 µl DNA extraction buffer (containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate) was added with 10 µl of Proteinase K to the faecal samples. The samples were put into the TissueLyser LT (Qiagen, DE) at maximum speed for 10 minutes and incubated at 56°C for an hour. Another 10 µl of Proteinase K was added to the samples and incubated at 56°C overnight. Thereafter the samples were centrifuged for 5 minutes at 12 000 rpm and the supernatant was transferred to 1.5 ml microcentrifuge tubes. A volume 180 µl of 5 M NaCl was added to the supernatant, vortexed for 30 seconds and centrifuged for 5 minutes at 13500 rpm. The supernatant was transferred to new 1.5 ml microcentrifuge tubes and 420 µl of cold isopropanol (Propan-2-ol) was added. The mixture was mixed by inverting the tubes fifty times slowly followed by centrifugation for 15 minutes at 13 500 rpm, at 4°C to precipitate the DNA. Subsequent to centrifugation, the supernatant was discarded, the DNA pellet was washed by adding 250 µl of 70% ethanol followed by being vortexed for 30 seconds and centrifuged for 5 minutes at 13500 rpm. The supernatant was discarded, and the washing step was repeated. After washing the DNA pellet, the Eppendorf tubes were left to air dry for 1 hour at room temperature for the evaporation of the ethanol. The DNA was dissolved in 200 µl of double distilled water. A NanoDrop spectrophotometer (Thermo Fischer, USA) was used to obtain the DNA concentration and the samples were stored at -20°C until use.

3.8. Conventional PCR

Conventional PCR was performed for the screening of the different protozoan parasites that include *Trypanozoon* spp., *T. congolense, T. gondii* and *Cryptosporidium* spp.

3.8.1. Amplification of *Trypanozoon* spp. DNA

Primers designed specifically to identify *Trypanozoon* species targeting the RIME gene was designed. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template. The reaction that was performed included a negative control of double distilled water and positive control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C ∞.

PCR products were electrophoresed using 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

3.8.2. Amplification of *T. congolense* DNA

Primers specific to *T. congolense* targeting the rP0 gene were designed. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template. The reaction that was performed included a negative control of double distilled water and positive control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C. PCR products were electrophoresed using 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

3.8.3. Amplification of *Cryptosporidium* spp. DNA

Primers specific to *Cryptosporidium* spp. targeting the HSP70 gene were designed. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template. The reaction that was performed included a negative control of double distilled water and positive control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C ∞. PCR products were electrophoresed using 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

3.8.4. Amplification of *T. gondii* DNA

Primers designed specifically to identify *T. gondii* targeting the B1 gene were designed. The reaction composed of a final volume of 25 µl which included 12.5 µl AmpliTaq Gold 360 Master mix (Applied Biosystems, USA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl GC enhancer, 8 µl of double distilled water and 2 µl of DNA template. The reaction that was performed included a negative control of double distilled water and positive

control. Using the ProFlex PCR System (Thermo Fisher Scientific, USA) the PCR conditions were as follow: Initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute and final elongation at 72°C for 7 minutes followed by a final hold at 4°C. PCR products were electrophoresed using 1% agarose electrophoresis gel stained with ethidium bromide and visualized under ultraviolet light using the ENDURO GDS Gel Documentation System (Labnet International, Inc., USA).

CHAPTER 4

RESULTS

4.1. HRM-Primer and gBlock design

For HRM-primer and gBlock design several NCBI Genbank sequences were downloaded as shown in table 4.1. and used for sequence alignment and for the design of the primers and gBlocks.

4.1.1. Sequence alignment

4.1.1.1. *Trypanosoma* spp.

The conserved regions can be seen in yellow and single nucleotide polymorphism can be seen in white and blue in figure 4.1. and 4.2. for *Trypanosoma* spp.

4.1.1.1. a). *Trypanozoon* spp.

The average length of the sequenced RIME gene fragments that was identified were 154 bp. The gene sequence identity ranged between 98-100% for the RIME gene as shown in table 4.1.

4.2.1.1. b). *T. congolense*

The average length of the sequenced rP0 gene fragments that was identified were 155 bp as shown in figure 4.2. The gene sequence identity was 100% identical for the rP0 gene as shown in table 4.2.

4.2.1.2. *Cryptosporidium* spp.

The conservered regions can be seen in yellow and single nucleotide polymorphism can be seen in white and blue in figure 4.3. for *Cryptosporidium* spp. The average length of the sequenced HSP70 gene fragments that was identified were 170 bp. The gene sequence identity ranged between 96-100% identical for the HSP70 gene as seen in table 4.2.

4.2.1.3. *T. gondii*

The conservered regions can be seen in yellow and single nucleotide polymorphism can be seen in white and blue in figure 4.4. The average length of the sequenced B1 gene fragments that was identified were 98 bp. The gene sequence identity ranged between 100% identical for the B1 gene as seen in table 4.1.

By incorporating the recommendations of Worjdacz & Lotte (2006) and Smith *et al.* (2009), the following primers indicated in Table 4.2. were designed for *Trypanosoma* spp., *Cryptosporidium* spp. and *T. gondii* after conducting sequence alignments.

Table 4.3. indicates the gBlocks that were designed after sequence alignment and primers were designed for *Trypanosoma* spp., *Cryptosporidium* spp. and *T. gondii.*

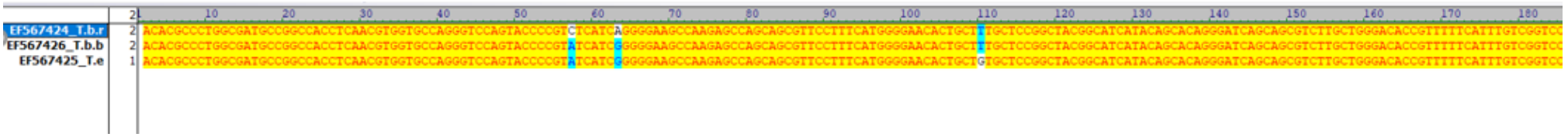

Figure 4.1: Sequence alignment of *Trypanozoon* spp. and the fragment size that was identified. The sequences contain and indicate the conserved and the different single nucleotide polymorphisms of the sequences to identify between species.

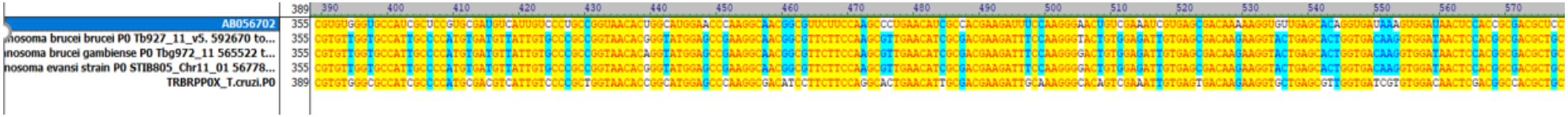

Figure 4.2: Sequence alignment of *T. congolense* and the fragment size that was identified. The sequences contain and indicate the conserved and the different single nucleotide polymorphisms of the sequences.

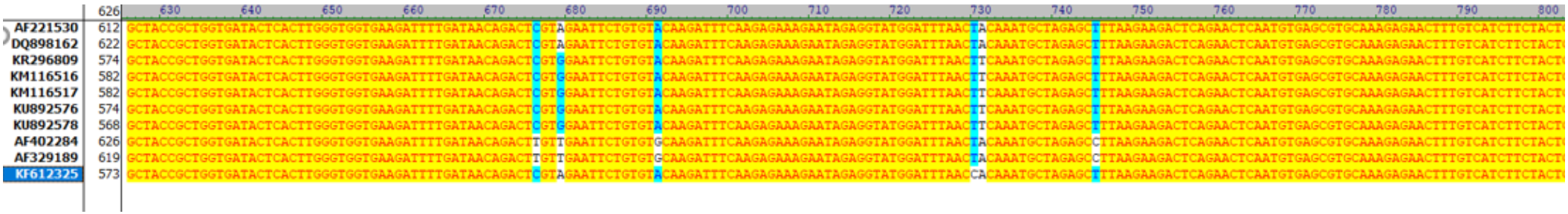

Figure 4.3: Sequence alignment of *Cryptosporidium* spp. and the fragment size that was identified. The sequences contain and indicate the conserved and the different single nucleotide polymorphisms of the sequences between species.

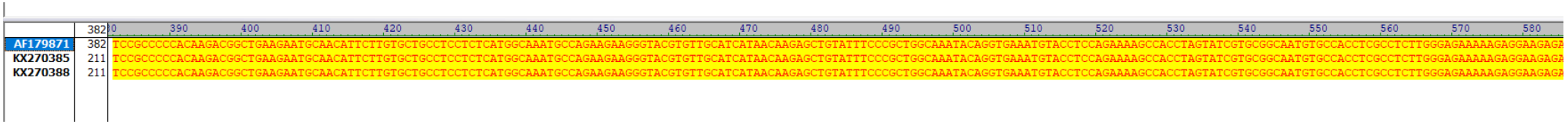

Figure 4.4: Sequence alignment of *T. gondii*. and the fragment size that was identified. The sequences contain and indicate the conserved sequences to identify the species.

Table 4.1: BLASTn results of target gene regions for sequence alignment and primer design

Gene	Blast description	Maximum score	Total score	Query cover	E. value	Identity	Accession
RIME	*T. b. rhodesiense*	944	944	100%	0	100%	EF567424
	T. b. brucei	928	928	100%	0	99.41%	EF567426
	T. evansi	889	889	99%	0	98.24%	EF567425
rP0	*T. congolense*	1912	1912	100%	0	100%	AB056702
	T. cruzi	323	388	84%	1e-86	80.02%	L15558
	T. b. gambiense	579	4959	97%	1e-163	83.86%	XM_011781070
HSP70	*C. parvum*	3578	3578	100%	0	100%	AF221530
	C. parvum	3504	3504	98%	0	99.79%	DQ898162
	C. hominis	3330	3330	98%	0	98.31%	KR296809
	C. hominis	3310	3310	97%	0	98.20%	KM116516
	C. hominis	3310	3310	97%	0	98.20%	KM116517
	C. hominis	3306	3306	97%	0	98.30%	KU892576
	C. hominis	3349	3349	98%	0	98.26%	KU892578
	C. erinacei	3325	3325	97%	0	98.63%	KF612325
	C. meleagridis	3142	3142	98%	0	96.24%	AF402284
	C. meleagridis	3125	3125	98%	0	96.27%	AF329189
B1	*T. gondii*	4089	4089	100%	0	100%	AF179871
	T. gondii	1592	1592	36%	0	100%	KX270388
	T. gondii	1592	1592	36%	0	100%	KX270385

Table 4.2: HRM-qPCR Primers

Genus	Target Gene	Primer Sequence (5'-3')	Product size (bp)	Length (bp)	Tm (°C)	%GC	Reference
Cryptosporidium spp.	HSP70	CrHSP70F1: GCTACCGCTGGTGATACTCACTTG	170	25	59.9	56	Morgan *et al*. (2001)
Cryptosporidium spp.	HSP70	CrHSP70R1: GATGACAAAGTTCTCTTTGCACG	170	23	53.3	43.5	
Trypanozoon spp.	RIME	TrRIMEF1: ACACGCCCTGGCGATGCCGGCCACC	155	25	77.7	76	Njiru *et al*. (2008)
Trypanozoon spp.	RIME	TrRIMER1: CAAGACGCTGCTGATCCCTGTGC	155	23	62.7	60.9	
Trypanosoma congolense	rP0	TrP0F1: CGTGTTGGTGCCATTGCCCC	112	20	62.7	65	Kuboki *et al*. (2003)
Trypanosoma congolense	rP0	TrP0R1: CGTCGCCGTGGAGTTATCC	112	19	55.0	63.2	
Toxoplasma gondii	B1	TG1586HRM1: CGCAGAGATGATACAGAGACGT	98	22	52.1	50	Cardona *et al*. (2011)
Toxoplasma gondii	B1	TG1586HRM2: GGTTGTTGAGGTCCGAAAGG	98	20	52	55	

Table 4.3: gBlock design and alignment

gBlock	Sequence of gBlock
gblock 1	***C. parvum* (HSP70 gene)** GCTACCGCTGGTGATACTCACTTGGGTGGTGAAGATTTTGATAACAGACTCGTAGAATTCTGTGTACAAGATTTCAAGAGAAAGAATAGAGGTATGGATTTAACTACA AATGCTAGAGCTTTAAGAAGACTCAGAACTCAATGTGAGCGTGCAAAGAGAACTTTGTCATC ***T. b. rhodesiense* (RIME gene)** ACACGCCCTGGCGATGCCGGCCACCTCAACGTGGTGCCAGGGTCCAGTACCCCGTCTCATCAGGGGAAGCCAAGAGCCAGCAGCGTTCCTTTCATGGGGAACAC TGCTTTGCTCCGGCTACGGCATCATACAGCACAGGGATCAGCAGCGTCTTG **T. cruzi (rP0 gene)** CGTGTGGGCGCCATCGCCCCATGCGACGTCATTGTCCCCGCTGGTAACACCGGCATGGAGCCCAAGGCGACATCCTTCTTCCAGGCACTGAACATTGCGACGAAG ATTGCAAAGGGCACAGTCGAAATTGTGAGTGACAAGAAGGTGCTGAGCGTTGGTGATCGTGTGGACAACTCGACGGCCACG
gBlock 2	***C. hominis* (HSP70 gene)** GCTACCGCTGGTGATACTCACTTGGGTGGTGAAGATTTTGATAACAGACTCGTGGAATTCTGTGTACAAGATTTCAAGAGAAAGAATAGAGGTATGGATTTAACTTCA AATGCTAGAGCTTTAAGAAGACTCAGAACTCAATGTGAGCGTGCAAAGAGAACTTTGTCATC ***T. b. brucei* (RIME gene)** ACACGCCCTGGCGATGCCGGCCACCTCAACGTGGTGCCAGGGTCCAGTACCCCGTATCATCGGGGGAAGCCAAGAGCCAGCAGCGTTCCTTTCATGGGGAACAC TGCTTTGCTCCGGCTACGGCATCATACAGCACAGGGATCAGCAGCGTCTTG ***T. b. brucei*. (rP0 gene)** CGTGTTGGTGCCATTGCCCCATGTGATGTTATTGTGCCCGCCGGTAACACGGGTATGGAGCCGAAGGCAACGGCGTTCTTCCAAGCGTTGAACATCGCGACGAAG ATTTCCAAGGGTACTGTGGAGATTGTGAGCGACAAGAAGGTACTGAGCACTGGTGACAAGGTGGATAACTCCACGGCGACG

gBlock 3	***C. meleagridis* (HSP70 gene)** GCTACCGCTGGTGATACTCACTTGGGTGGTGAAGATTTTGATAACAGACTTGTTGAATTCTGTGTGCAAGATTTCAAGAGAAAGAATAGAGGTATGGATTTAACTACA AATGCTAGAGCCTTAAGAAGACTCAGAACTCAATGTGAGCGTGCAAAGAGAACTTTGTCATC ***T. b. gambiense* (rP0 gene)** CGTGTTGGTGCCATTGCCCCATGTGATGTTATTGTGCCCGCCGGTAACACAGGTATGGAGCCGAAGGCAACGGCGTTCTTCCAAGCGTTGAACATCGCGACGAAG ATTTCCAAGGGGACTGTGGAGATTGTGAGCGACAAGAAGGTACTGAGCACTGGTGACAAGGTGGATAACTCCACGGCGACG
gBlock 4	***C. erinacei* (HSP70 gene)** GCTACCGCTGGTGATACTCACTTGGGTGGTGAAGATTTTGATAACAGACTCGTAGAATTCTGTGTACAAGATTTCAAGAGAAAGAATAGAGGTATGGATTTAACCACA AATGCTAGAGCTTTAAGAAGACTCAGAACTCAATGTGAGCGTGCAAAGAGAACTTTGTCATC ***T. b. evansi* (RIME gene)** ACACGCCCTGGCGATGCCGGCCACCTCAACGTGGTGCCAGGGTCCAGTACCCCGTATCATCGGGGGAAGCCAAGAGCCAGCAGCGTTCCTTTCATGGGGAACAC TGCTGTGCTCCGGCTACGGCATCATACAGCACAGGGATCAGCAGCGTCTTG ***T. b. evansi* (rP0 gene)** CGTGTTGGTGCCATTGCCCCATGTGATGTTATTGTGCCCGCCGGTAACACGGGTATGGAGCCGAAGGCAACGGCGTTCTTCCAAGCGTTGAACATCGCGACGAAG ATTTCCAAGGGGACTGTGGAGATTGTGAGCGACAAGAAGGTACTGAGCACTGGTGACAAGGTGGATAACTCCACGGCGACG
gBlock 5	***T. congolense* (rP0 gene)** CGTGTGGGTGCCATCGCTCCGTGCGATGTCATTGTCCCTGCCGGTAACACTGGCATGGAACCCAAGGCAACGGCGTTCTTCCAAGCCCTGAACATCGCCACGAAG ATTTCCAAGGGAACTGTCGAAATCGTGAGCGACAAAAAGGTGTTGAGCACAGGTGATAAAGTGGATAACTCCACCGCGACGC
gBlock 6	***Toxoplasma gondi*i (Toxo stain I-B1 gene)** GATGCTCAAAGTCGACCGCGAGATGCACCCGCAGAAGAAGGGCTGACTCGAACCAGATGTGCTAAAGGCGTCATTGCTGTTCTGTCCTATCGCAACGGAGTTCTTC CCAGACGTGGATTTCCGTTGGTTCGCAGAGATGATACAGAGACGTGTCATCAGGACAAGGTTGGTCGCTTAATTTTCTGTATATAGCATTTTTAGAATGCACCTTTCG GACCTCAACAACCGTGCAAAAGGATCGCCACCTG

4.3. HRM-qPCR Optimization

4.3.1. Annealing temperature and target gene optimization

There is a difference in the CT values between *Trypanozoon* spp. (RIME gene), *T. congolense* (rP0 gene), *Cryptosporidium* spp. (HSP70 gene) and *T. gondii* (B1 gene).

4.3.1.1.a) Subgenus *Trypanozoon* HRM-qPCR

For all the *Trypanozoon* spp. samples it was found to be ± 85°C as seen in table 4.4., where a difference can also be seen between species in the same subgenus. Figure 4.5. to figure 4.7. also indicate the same result between the same species samples in the same subgenus for gBlock 1, 2 and 4.

Table 4.4: The subgenus *Trypanozoon* (RIME gene) and the melt curve temperatures (°C) (CT values) at different concentrations.

Genus	gBlock	Concentration	CT value (°C)
Trypanozoon (RIME gene)	1	10^{-1}~1 ng/µl	85.598
		10^{-2}	85.396
		10^{-3}	85.291
		10^{-4}	85.193
		Negative control	0
	2	10^{-1}	85,163
		10^{-2}	85,203
		10^{-3}	85,124
		10^{-4}	85,087
		Negative control	0
	4	10^{-1}	85,439
		10^{-2}	85,439
		10^{-3}	85,439
		10^{-4}	85,442
		Negative control	0

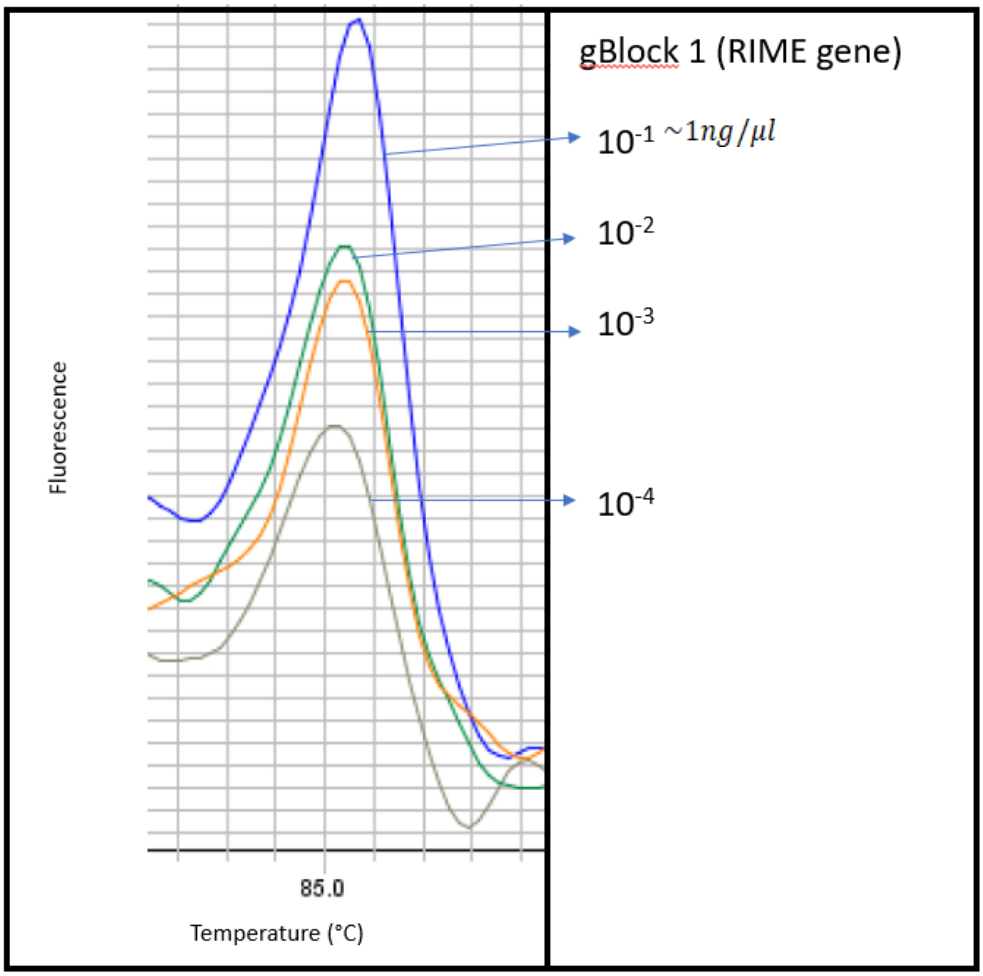

Figure 4.5: Derivative Melt curve of gBlock 1 for the RIME gene at different concentrations

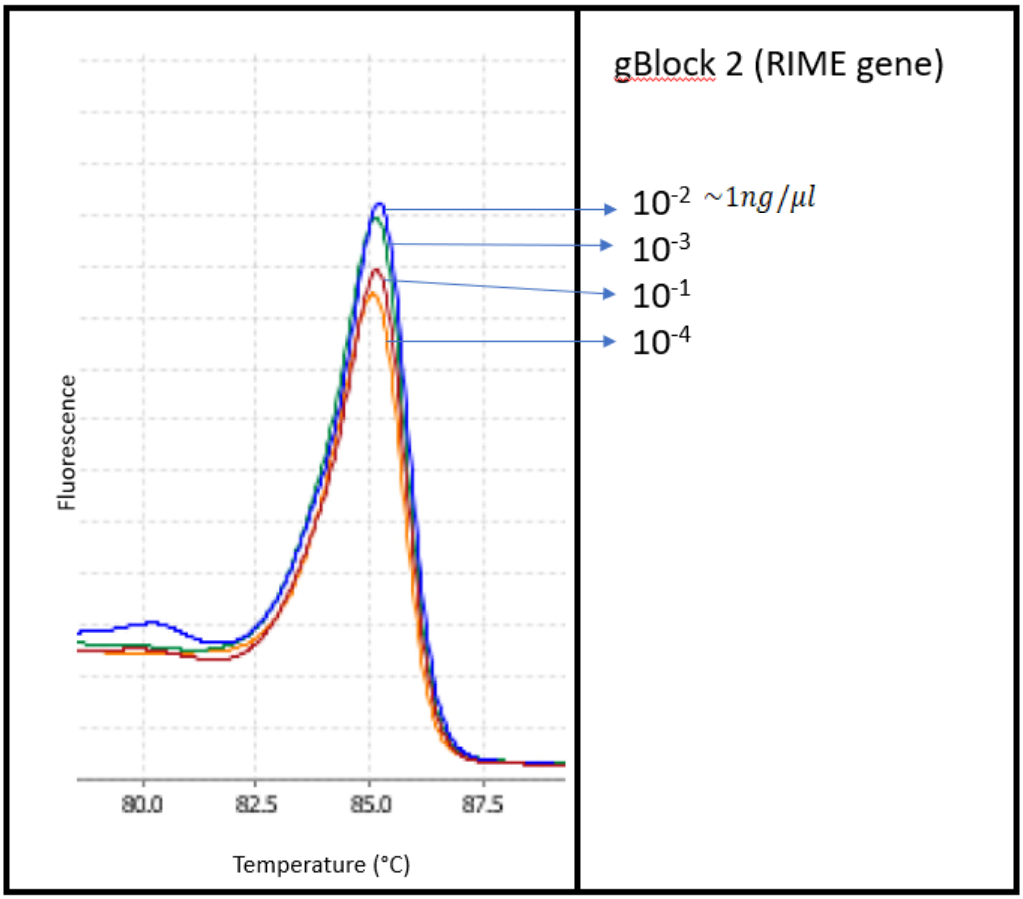

Figure 4.6: Derivative Melt curve of gBlock 2 for the RIME gene at different concentrations.

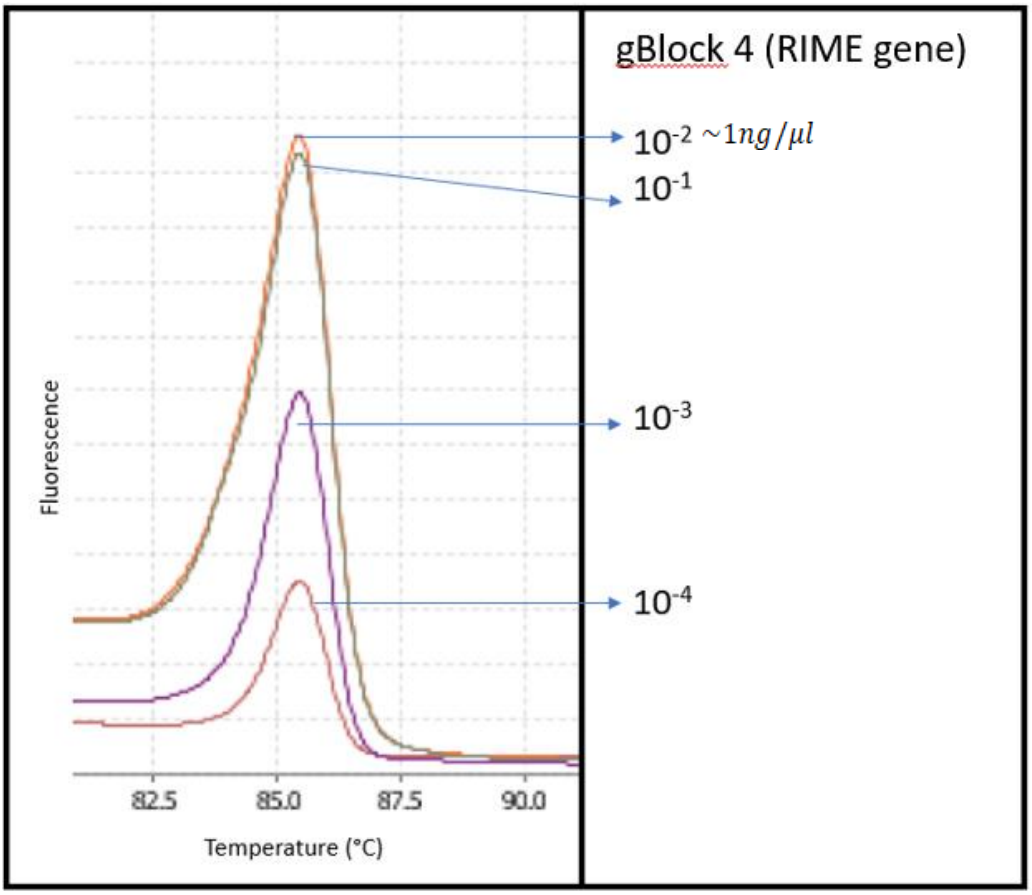

Figure 4.7: Derivative Melt curve of gBlock 4 for the RIME gene at different concentrations.

4.3.1.1.b) *T. congolense* HRM-qPCR

The melting temperature for *T. congolense* was optimal at $\pm$83°C as seen in table 4.5. with few differences. Figure 4.8 to figure 4.12 indicated the same results between the same species samples for gBlock 1, 2, 3 and 4.

Table 4.5: *T. congolense* (rP0 gene) and the melt curve temperatures (°C) (CT values) at different concentrations.

Genus	gBlock	Concentration	CT value (°C)
T. congolense (rP0 gene)	1	10^{-1}~1 ng/µl	84.173
		10^{-2}	84.071
		10^{-3}	83.769
		10^{-4}	83.870
		Negative control	0
	2	10^{-1}	83,150
		10^{-2}	83,30
		10^{-3}	83,149
		10^{-4}	83,111
		Negative control	0
	3	10^{-1}	83.970
		10^{-2}	83.560
		10^{-3}	83.464
		10^{-4}	83.565
		Negative control	0
	4	10^{-1}	83,387
		10^{-2}	83,347
		10-3	83,347
		10-4	83,27
		Negative control	0
	5	10^{-1}	82.960
		10^{-2}	82.859
		10-3	83.162
		10-4	83,767
		Negative control	0

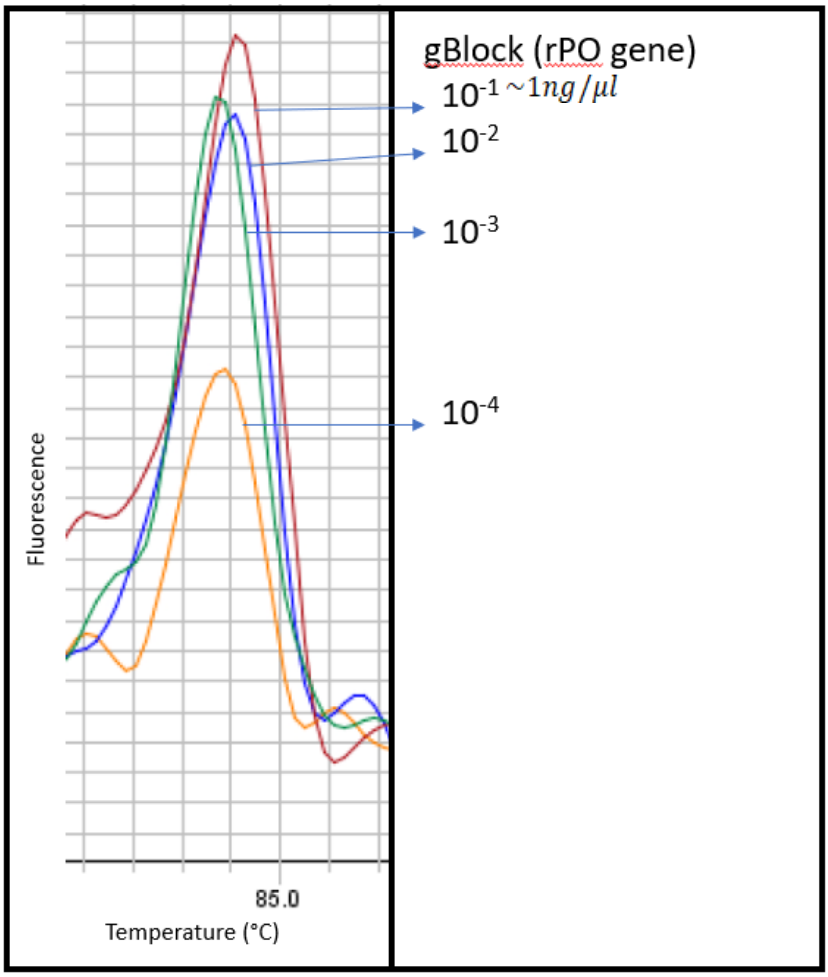

Figure 4.8: Derivative Melt curve of gBlock 1 for the rP0 gene at different concentrations.

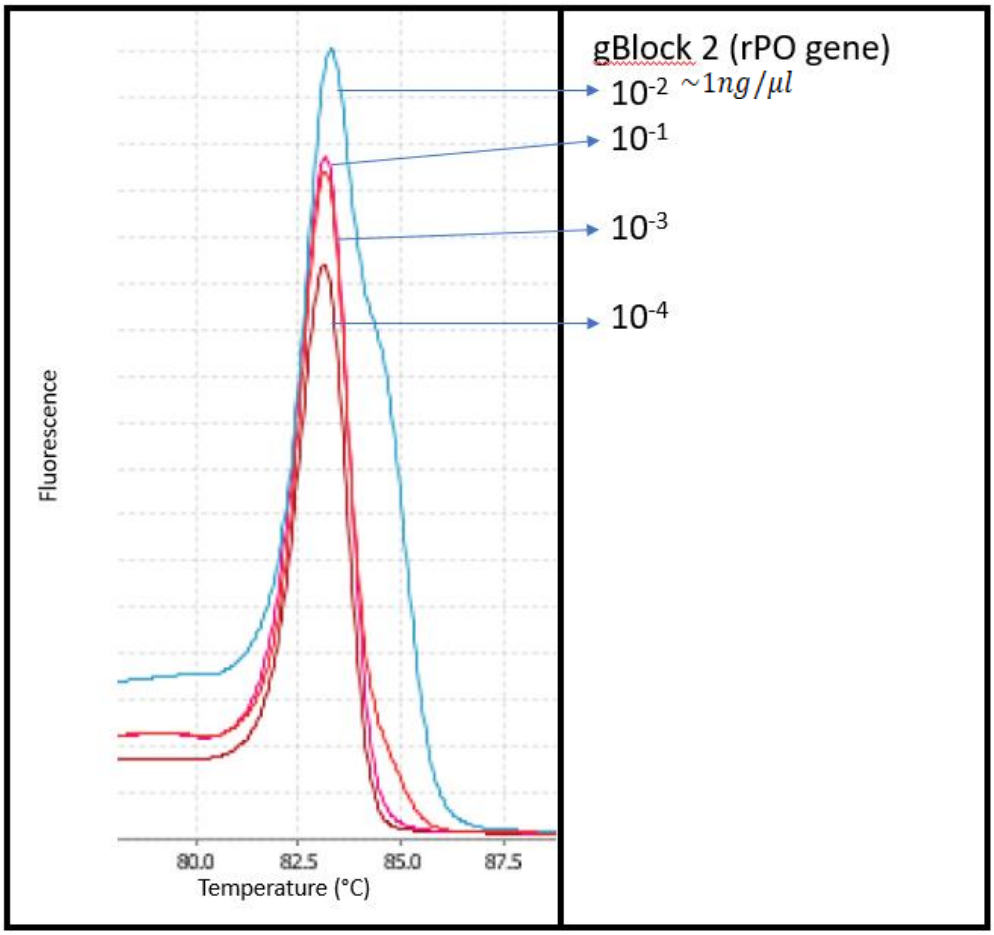

Figure 4.9: Derivative Melt curve of gBlock 2 for the rP0 gene at different concentrations.

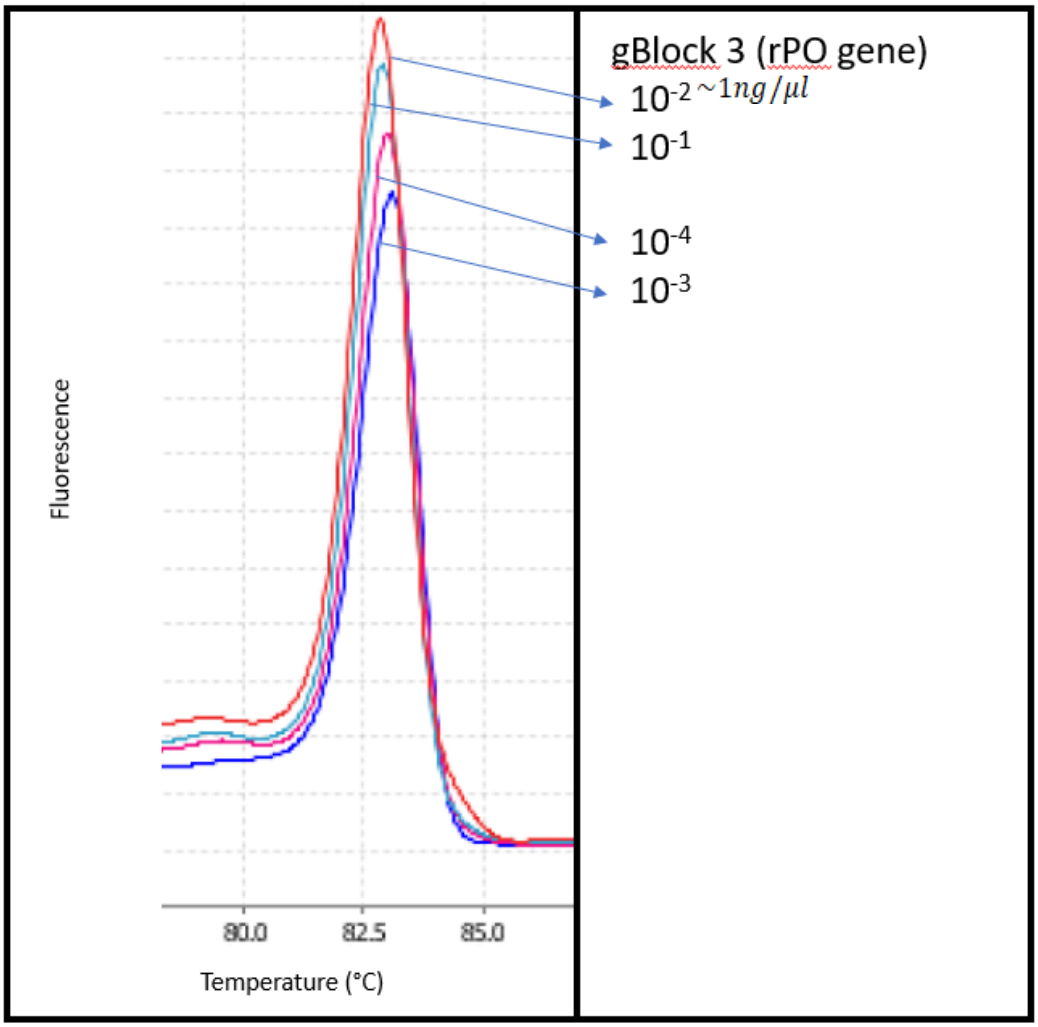

Figure 4.10: Derivative Melt curve of gBlock 3 for the rP0 gene at different concentrations.

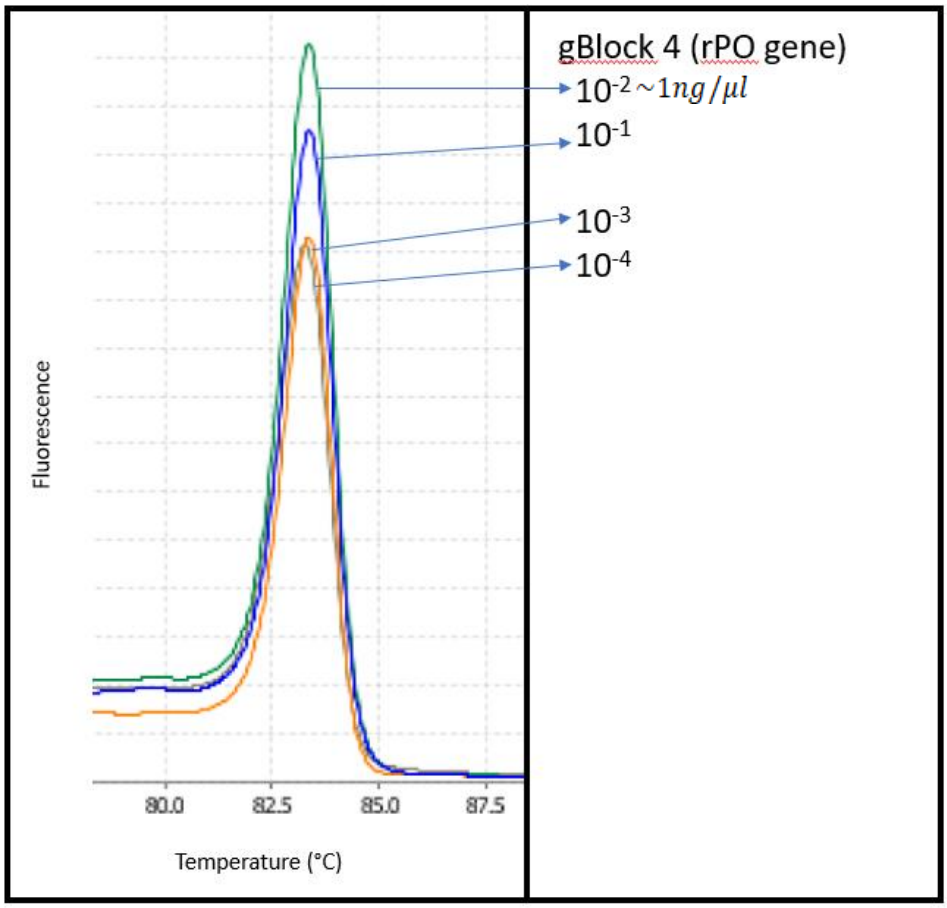

Figure 4.11: Derivative Melt curve of gBlock 4 for the rP0 gene at different concentrations.

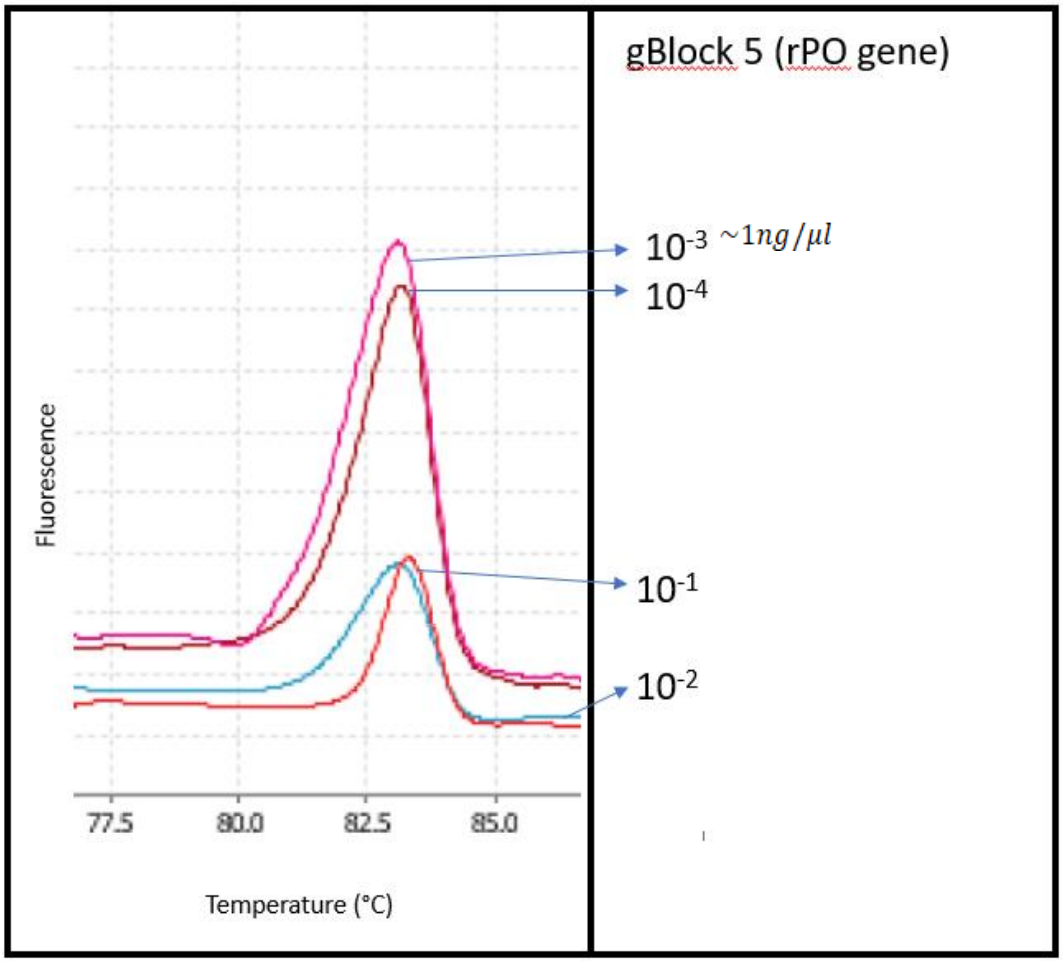

Figure 4.12: Derivative Melt curve of gBlock 5 for the rP0 gene at different concentrations.

4.3.1.2. *Cryptosporidium* spp. HRM-qPCR

The melting temperature for *Cryptosporidium* spp. samples were optimal at ±76°C as seen in table 4.6., where a difference was seen between species in the same genus. Figure 4.13. to figure 4.16. indicated the same results between the same species samples in the same genus.

Table 4.6: *Cryptosporidium* spp. (HSP70 gene) and the melt curve temperatures (°C) (CT values) at different concentrations.

Genus	gBlock	Concentration	CT value (°C)
Cryptosporidium (HSP70 gene)	1	10^{-1}~1 ng/µl	76,341
		10^{-2}	76,262
		10^{-3}	76,223
		10^{-4}	76,265
		Negative control	0
	2	10^{-1}	76,5
		10^{-2}	76,539
		10^{-3}	76,579
		10^{-4}	76,579
		Negative control	0
	3	10^{-1}	76,698
		10^{-2}	76,737
		10^{-3}	76,738
		10^{-4}	76,383
		Negative control	0
	4	10^{-1}	76,659
		10^{-2}	76,658
		10^{-3}	76,42
		10^{-4}	76,383
		Negative control	0

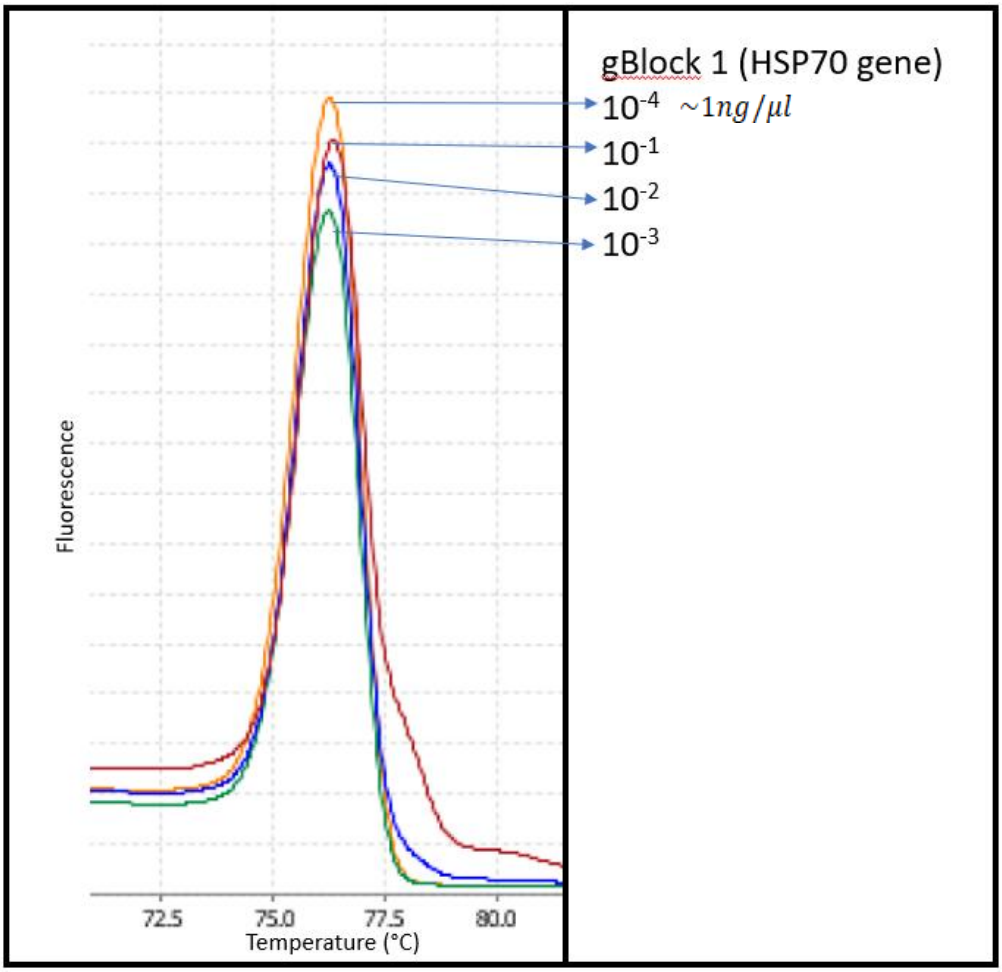

Figure 4.13: Derivative Melt curve of gBlock 1 for the HSP70 gene at different concentrations.

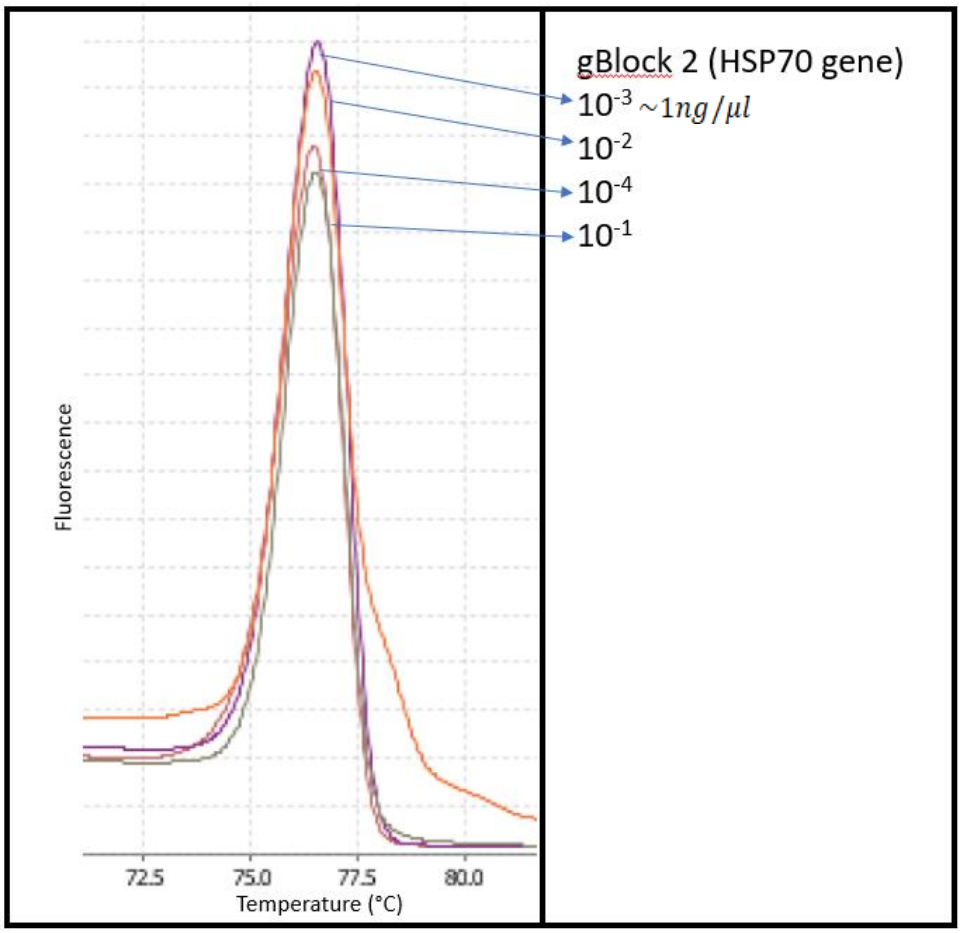

Figure 4.14: Derivative Melt curve of gBlock 2 for the HSP70 gene at different concentrations.

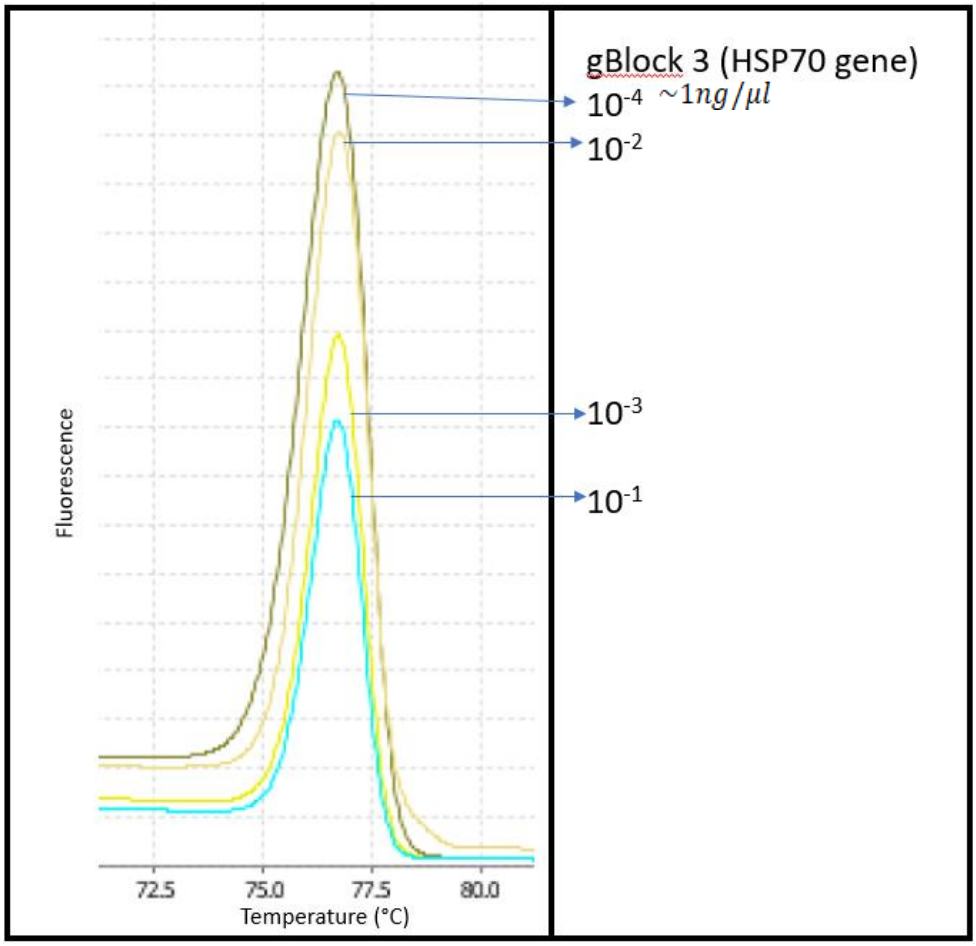

Figure 4.15: Derivative Melt curve of gBlock 3 for the HSP70 gene at different concentrations.

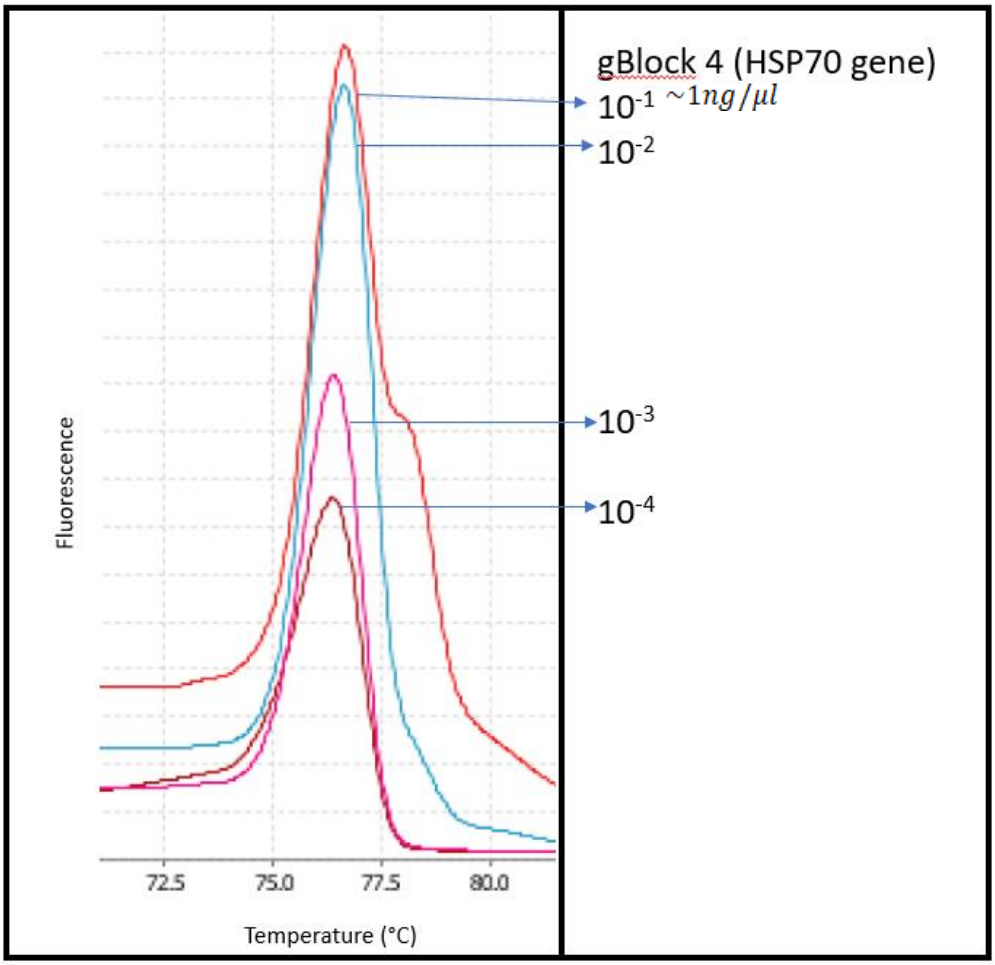

Figure 4.16: Derivative Melt curve of gBlock 4 for the HSP70 gene at different concentrations.

4.3.1.3. *T. gondii* HRM-qPCR

For all the *T. gondii* samples the optimum melt curve temperature was found to be ± 81°C as seen in Table 4.7 and Figure 4.17.

Table 4.7: *T. gondii* (B1 gene) and the melt curve temperatures (°C) (CT values) at different concentrations.

Genus	gBlock	Concentration	(CT value) (°C)
Toxoplasma gondii (B1 gene)	6	10^{-1}~1 ng/µl	81,469
		10^{-2}	81,469
		10^{-3}	81,266
		10^{-4}	81,266
		Negative control	0

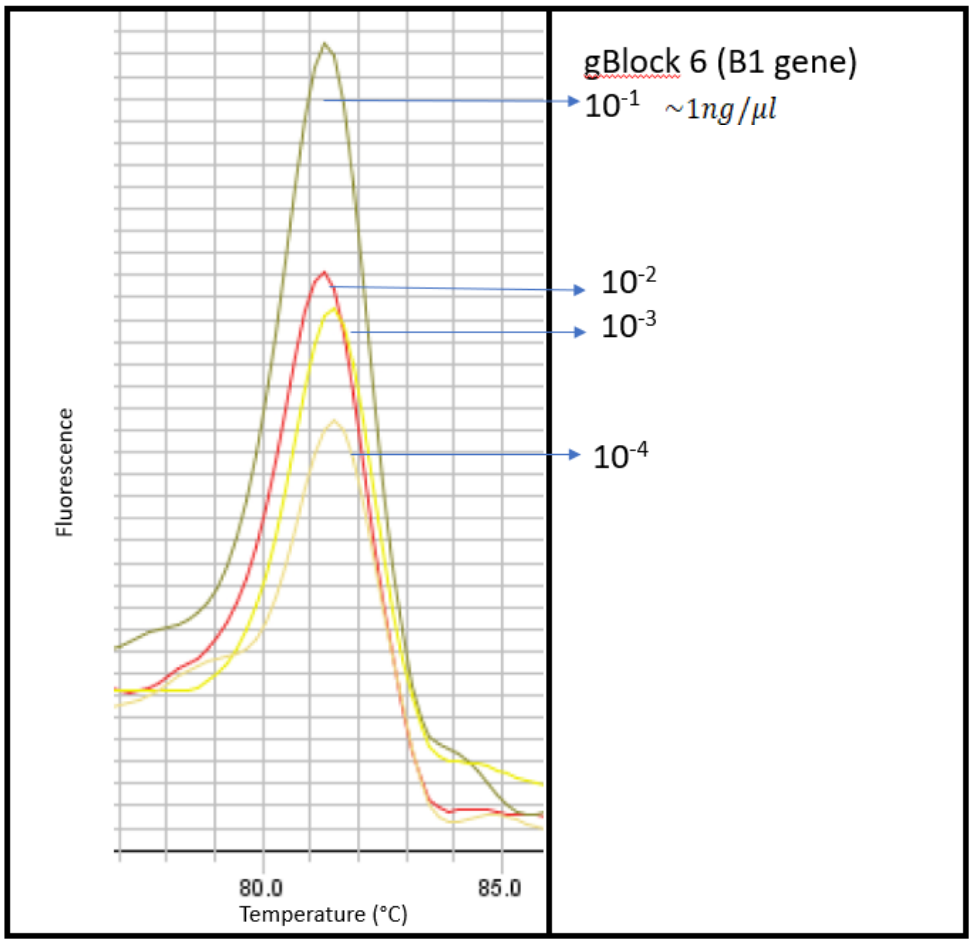

Figure 4.17: Derivative Melt curve of gBlock 6 for the B1 gene at different concentrations.

4.3.2. Sensitivity

The detection limit was tested by a ten-fold dilution series of the designed gBlocks of *Trypanozoon* spp., *T. congolense, Cryptosporidium* spp. and *T. gondii*. The dilution series started with 10^{-1} which is equivalent to ~1 ng/µl. The detection limit is indicated by amplification plots as seen in Figure 4.18, 4.19, 4.20 and 4.21, respectively, showing RIME HRM-qPCR for subgenus *Trypanozoon* species, rP0 HRM-qPCR for *T. congolense*, HSP70 HRM-qPCR *Cryptosporidium* spp. and B1 HRM-qPCR for *T. gondii* .

4.3.2.1a) Subgenus *Trypanozoon* spp.

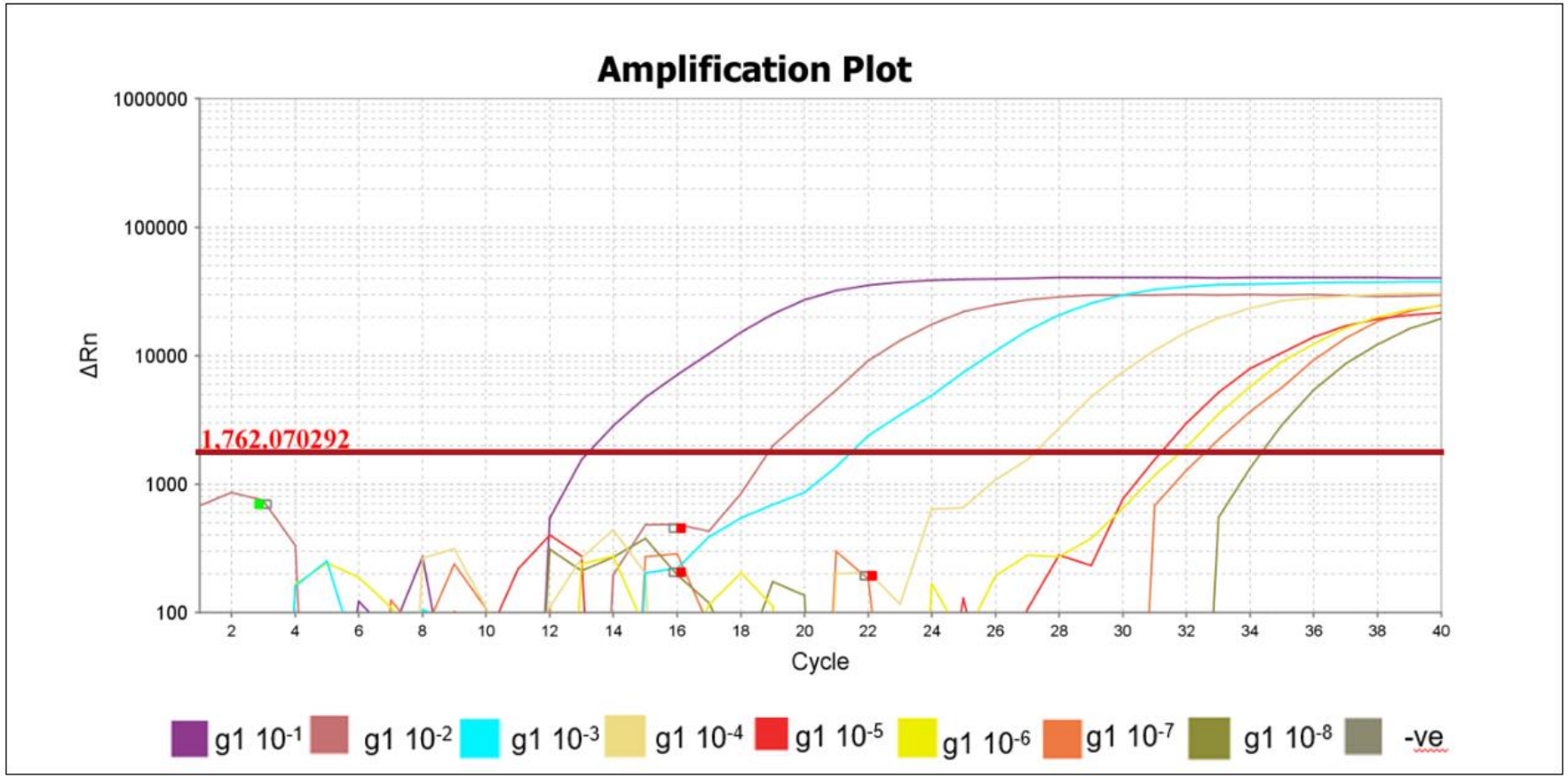

Figure 2.18: Amplification plot for *Trypanozoon* subgenus (Dilutions 10^{-1}~*1 ng/µl*)

4.3.2.1b) *T. congolense*

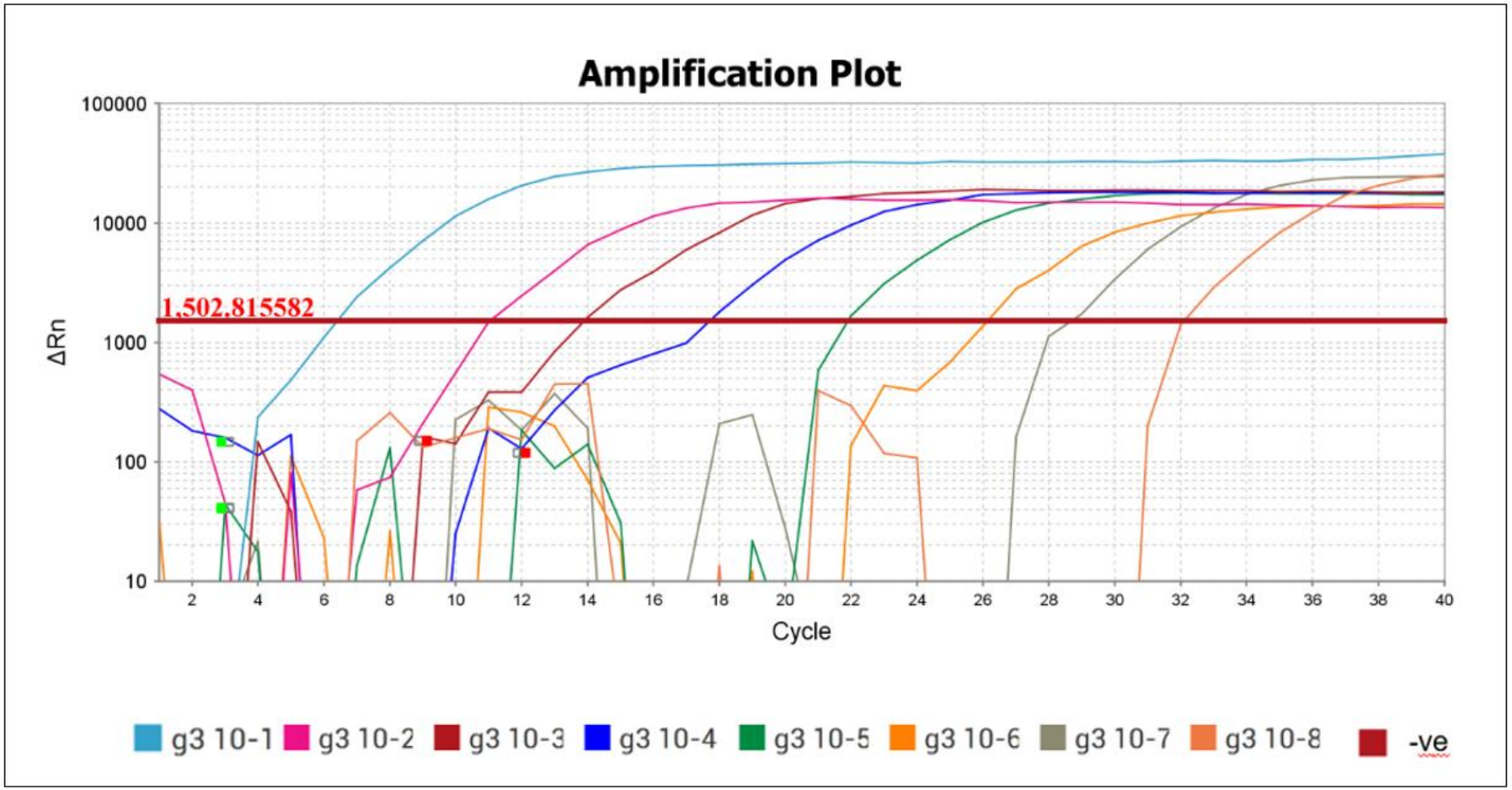

Figure 4.19: Amplification plot for *T. congolense* (Dilutions $10^{-1} \sim 1ng/\mu l$)

4.3.2.2. *Cryptosporidium* spp.

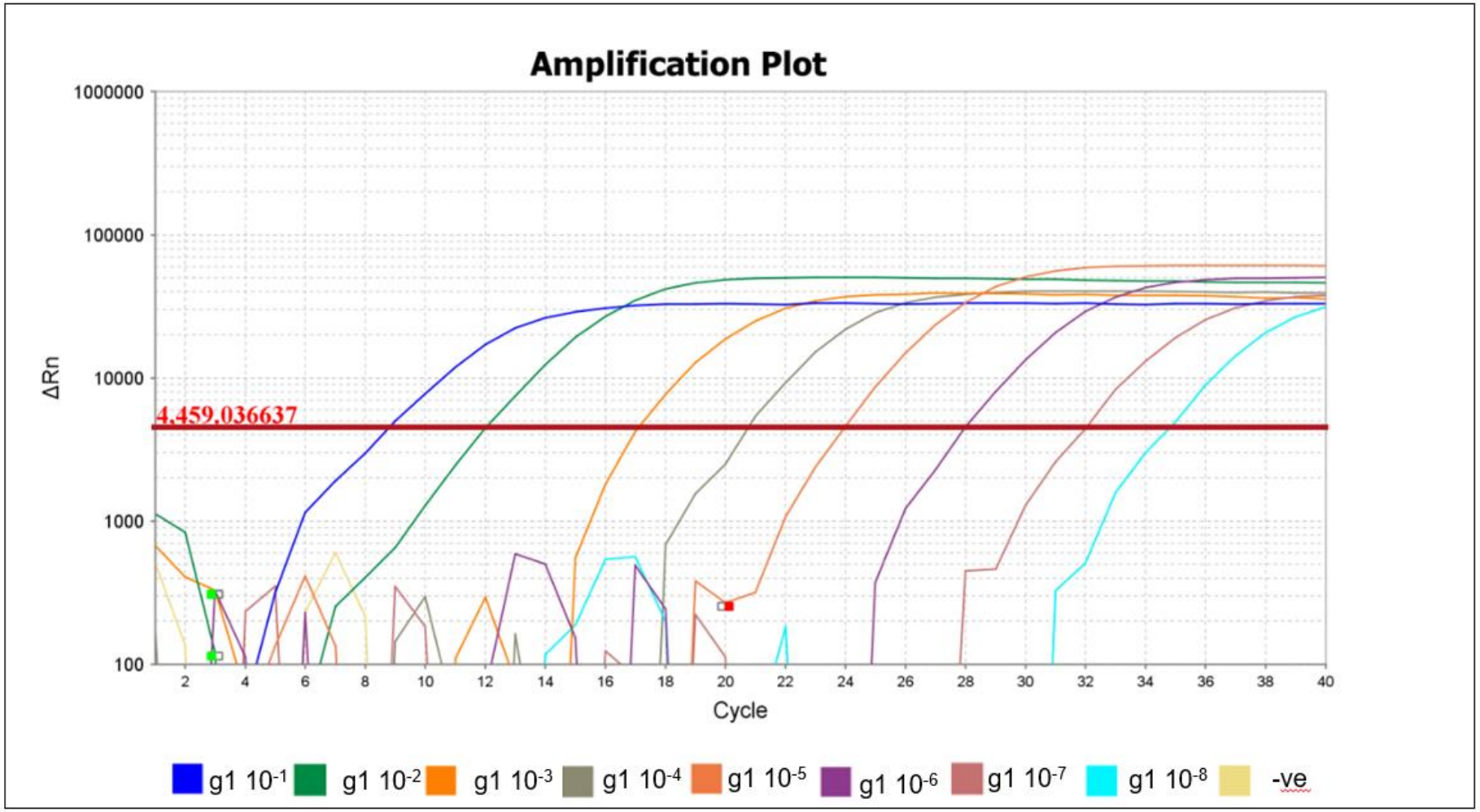

Figure 4.20: Amplification plot for *Cryptosporidium* spp. (Dilutions 10^{-1} $\sim 1ng/\mu l$)

4.3.2.3. *T. gondii*

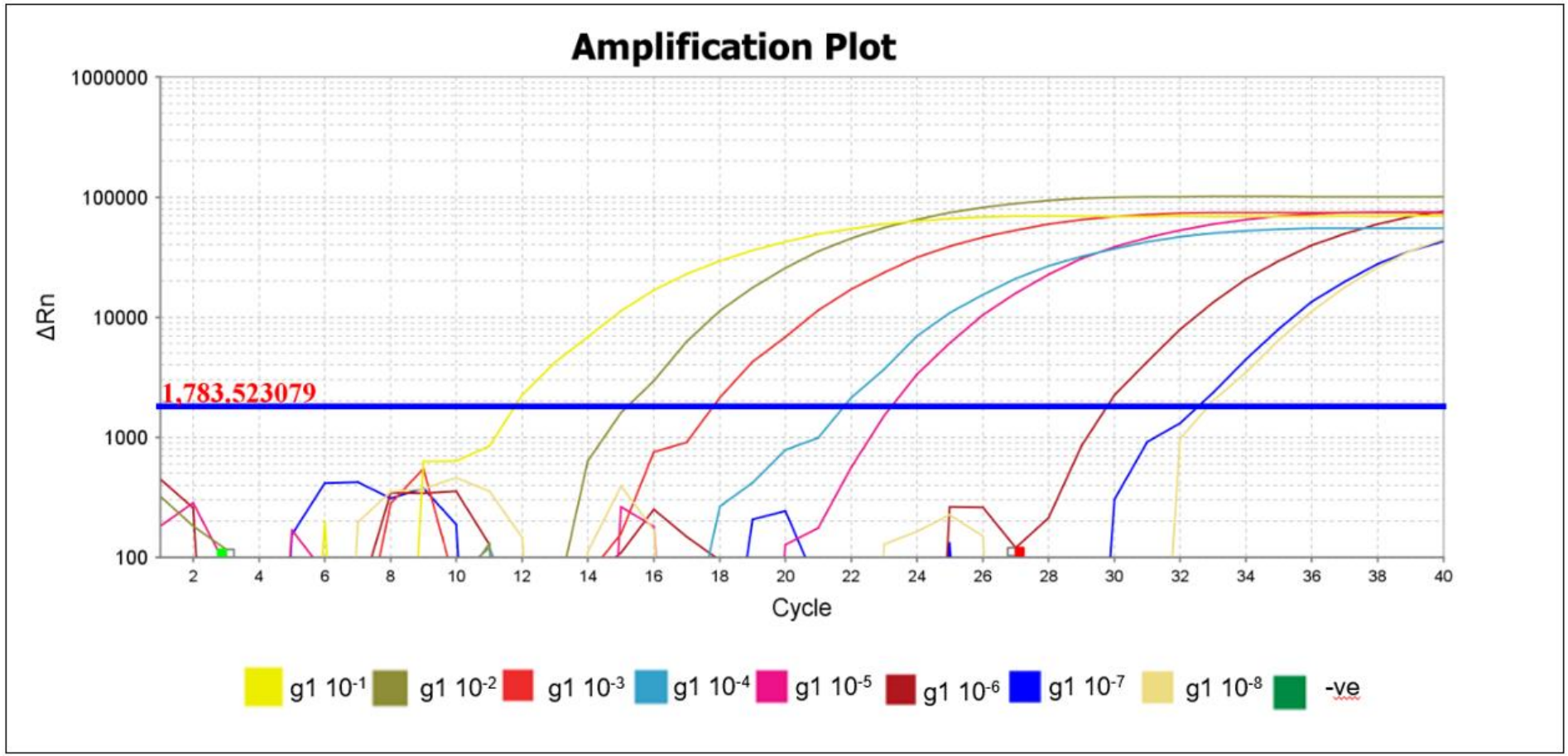

Figure 4.21: Amplification plot for *Toxoplasma gondii* (Dilutions 10^{-1} $\sim 1ng/\mu l$)

4.4. HRM-qPCR analysis

4.4.1.a) Subgenus *Trypanozoon* screening of field samples with HRM-qPCR

The screening of cattle and tsetse fly samples with the RIME target gene shown in Table 4.8. Fourteen positive field samples were indicated with sample ID: R1, R2, R3, R4, R6, R7, T1, T2, T3, T5, T6, T8, T9 and T10.

Table 4.8: The screening of *Trypanozoon* spp. field samples and their CT values

Sample	Temperature (°C) (CT values)				
R1	85.252	-	-	-	-
R2	85.677	-	-	-	-
R3	85.479	-	-	-	-
R4	85.232	-	-	-	-
R5	-				
R6	85.311	-	-	-	-
R7	85.615	-	-	-	-
T1	85.716	-	-	-	-
T2	85.919	-	-	-	-
T3	85.311	-	-	-	-
T4	-				
T5	85.836	-	-		
T6	85.836	-	-	-	-
T7	-	-	-	-	-
T8	85.634	-	-	-	-
T9	85.431	-	-	-	-
T10	85.330	-	-	-	
+ve	85.192				
-ve	0				

4.4.1.b) *T. congolense* screening of field samples with HRM-qPCR

The screening of cattle and tsetse fly samples with the rP0 target gene shown in table 4.9. Fourteen positive field samples were indicated with sample ID: R1, R2, R3, R4, R5, R6, R7, T2, T3, T4, T5, T6, T8, and T10.

Table 4.9: The screening of *T. congolense* field samples and their CT values

Sample	Temperature (°C) (CT values)				
R1	83.582				
R2	83.481		-		
R3	83.684	-	-	-	
R4	83.886				
R5	83.886	-	-	-	-
R6	83.886	-	-	-	
R7	84.088				
T1	-	-	-		
T2	83.804	-	-	-	-
T3	84.006	-	-	-	-
T4	84.006				
T5	83.702	-	-	-	-
T6	84.006	-	-		
T7	-	-	-	-	-
T8	83.601	-	-		
T9	-	-	-	-	
T10	84.076		-		
+ve	83.671				
-ve	0				

4.4.2. *Cryptosporidium* spp. screening of field samples with HRM-qPCR

The screening of sheep and wild bird samples with the HSP70 target gene shown in Table 4.10. Five positive field samples were indicated with sample ID: C1, C4, C6, C7 and C9.

Table 4.10: The screening of *Cryptosporidium* spp. field samples and their CT values

Sample	Temperature (°C) (CT values)				
C1	76.985				
C2	-				
C3	-				
C4	76.985	-			
C5	-	-	-		
C6	76.980				
C7	76.980	-	-	-	-
C8	-				
C9	76.677	-	-	-	
C10	-	-			
V1	-				
V2	-				
V3	-				
V4	-				
V5	-				
V6	-				
V7	-				
-ve	0				

4.4.3. *T. gondii* screening of field samples with HRM-qPCR

The screening of sheep and wild bird samples with the B1 target gene shown in table 4.11. Four positive field samples were indicated with sample ID: C1, C3, C4 and C5.

Table 4.11: The screening of *T. gondii* field samples and their CT values

Sample	Temperature (°C) (CT values)			
C1	81.469	-		
C2	-			
C3	81.469			
C4	81.266			
C5	81.266			
C6	-			
C7	-			
C8	-			
C9	-			
C10	-			
V1	-	-	-	-
V2	-	-	-	
V3	-			
V4	-	-	-	-
V5	-			
V6	-	-	-	
V7	-	-		
-ve	0			

4.6. DNA extraction

DNA was extracted from the faecal samples using the salting out method (Rivero *et al,*. 2006). The spectrophotpmeter was used to quantify DNA samples and the concentrations varied from 12.9 ng/µL to 365.0 ng/µL.

4.7. Conventional PCR

4.7.1. RIME target gene of cattle blood and tsetse flies' samples

Amplification of the DNA targeting the RIME gene was successful with a PCR product size of 112 bp (Figure 4.22).

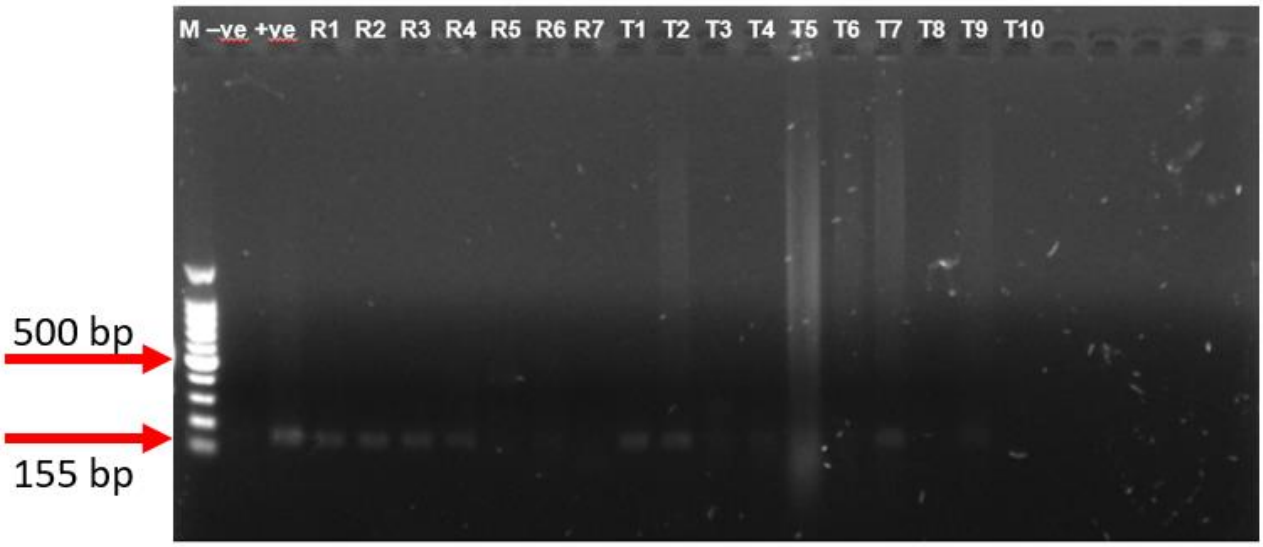

Figure 4.22: The 1% agarose electrophoresis gel showing PCR amplicons of 155 bp of cattle DNA amplified using the RIME gene. Sample marked as –ve is a negative control, and the sample +ve is a positive control. for the screening of extracted DNA samples, of cattle blood (R1-R7) and tsetse flies (T1-T10), targeting the RIME gene with an expected product size of 155 bp. Lane 1 (M): 100bp DNA molecular marker. Lane 2 (ve-): No template control. Lane 3 (ve+): *Trypanozoon* positive control. Lane 3, 4, 5, 6, 10, 11, 13, 14, 16 and 18 shows positive samples.

4.7.2. rP0 target gene of cattle blood and tsetse flies' samples

Amplification of the DNA targeting the rP0 gene was successful with a PCR product size of 112 bp (Figure 4.23).

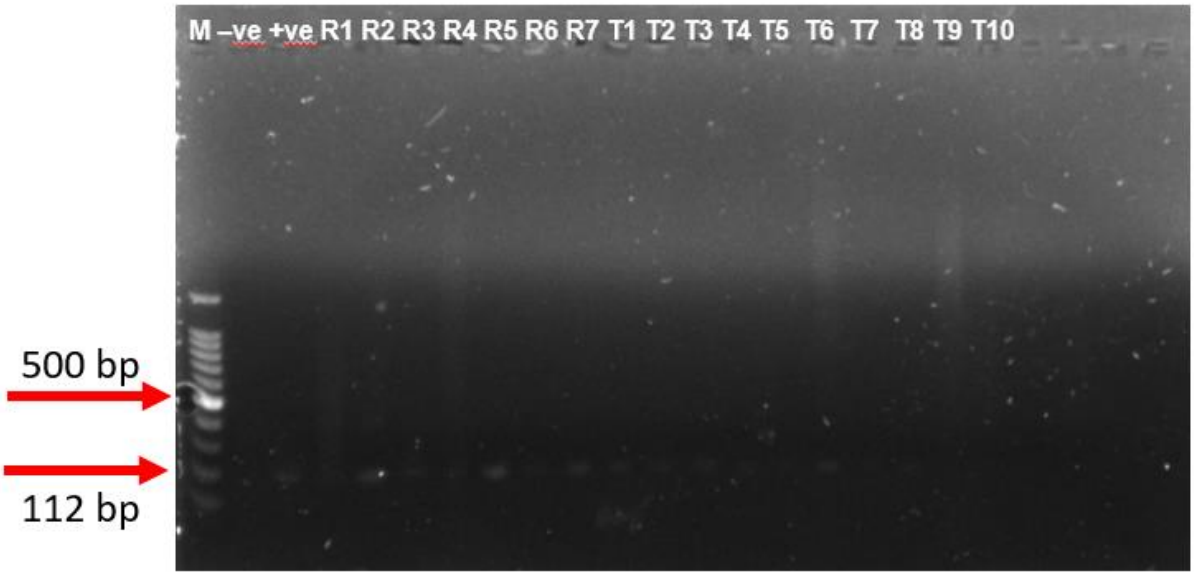

Figure 4.23: The 1% agarose electrophoresis gel for the screening of extracted DNA samples, of cattle blood (R1-R7) and tsetse flies (T1-T10), targeting the rP0 gene with an expected product size of 112 bp Lane 1 (M): 100bp DNA molecular marker. Lane 2 (ve-): No template control. Lane 3 (ve+): *T. congolense* positive control. Lane 4, 5, 7, 9, 10, 11, 12, 15 and 17 shows positive samples.

4.7.3. HSP70 target gene of sheep faecal and wild bird faecal samples

Amplification of the DNA targeting the HSP70 gene was successful with a PCR product size of 170 bp (Figure 4.24).

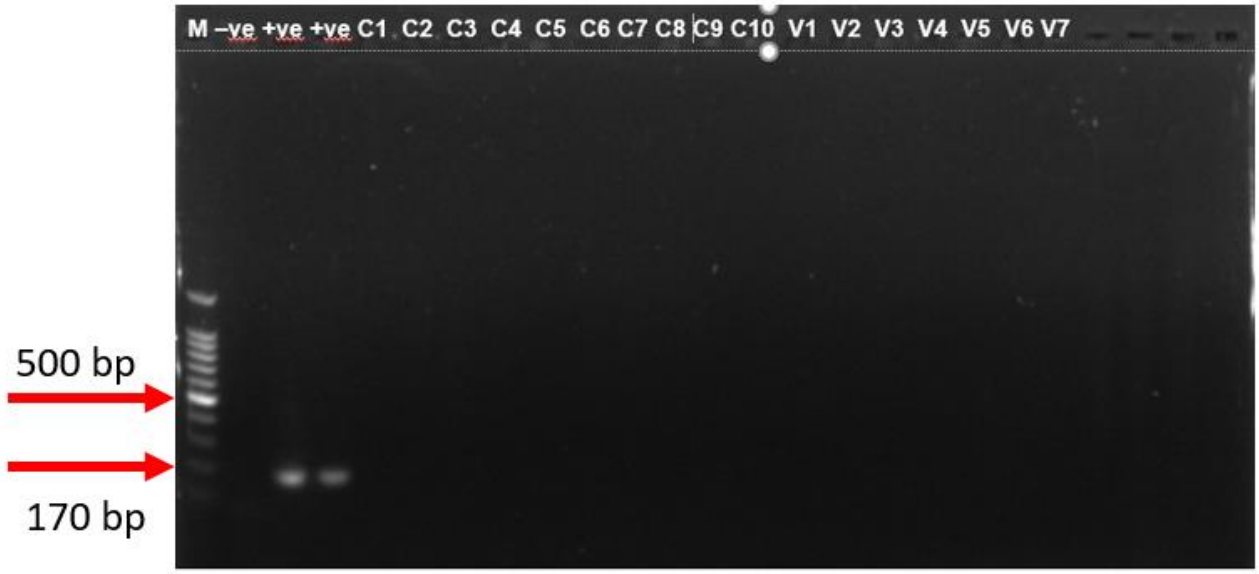

Figure 4.24: The 1% agarose electrophoresis gel for the screening of extracted DNA samples, of sheep (C1-C10) and wild birds (V1-V7), targeting the HSP70 gene with an expected product size of 170 bp Lane 1 (M): 100bp DNA molecular marker. Lane 2 (ve-): No template control. Lane 3 & 4 (ve+): *Cryptosporidium* spp. positive control.

4.7.4. B1 target gene of sheep faecal and wild bird faecal samples

Amplification of the DNA targeting the HSP70 gene was successful with a PCR product size of 98 bp (Figure 4.25).

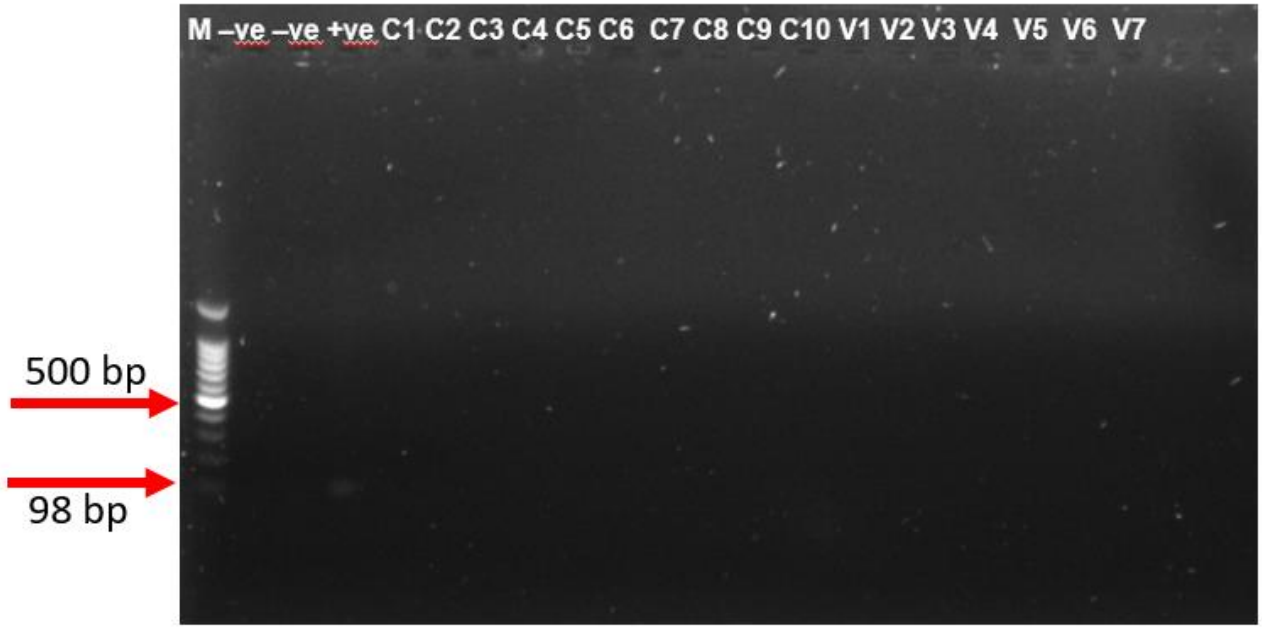

Figure 4.25: The 1% agarose electrophoresis gel for the screening of extracted DNA samples, of sheep (C1-C10) and wild birds (V1-V7), targeting the B1 gene with an expected product size of 98 bp. Lane 1 (M): 100bp DNA molecular marker. Lane 2 & 3 (ve-): No template control. Lane 3 (ve+): *Toxoplasma* positive control.

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

The HRM-qPCR is a rapid and simple assay combining real time-PCR analysis with high resolution melt analysis and is used for identification between DNA sequences. It is a cost-effective reaction method compared to other methods (Reed *et al*., 2007). The target region is amplified with the presence of a SYPO 9 Green Fluorescent nuclei acid stain in the HRM mastermix. The difference in the temperatures between the samples is formed due to sequence variation in homozygous samples (Life Technologies Corporation, 2010).

5.1.1. *Trypanosoma* spp.

5.1.1. a) *Trypanozoon* spp.

The RIME gene is expressed by subgenus *Trypanozoon* species including *T. b. gambiense, T. b. rhodesiense, T. b. brucei, T. evansi* and *T. equiperdum*. Njiru *et al.* (2008) successfully developed a loop-mediated isothermal amplification (LAMP) method based on RIME gene which successfully and specifically amplified the *T. b. gambiense, T. b. rhodesiense, T. b. brucei* and *T. evansi* DNA. In this study RIME gene sequence alignment has shown that the gene is conserved amongst subgenus *Trypanozoon* parasites i.e. *T. b. rhodesiense, T. b. brucei* and *T. evansi* with few single nucleotide polymorphisms (Figure 4.1). This is an indication that target gene is a good candidate for the development of a diagnostic assay for detection of subgenus *Trypanozoon* species, hence in the current study, HRM-qPCR assay was designed from it.

The optimization experiments consistently indicated the melt curve peak for positive HRM-qPCR reaction for amplification of subgenus *Trypanozoon* species RIME gene (gBlock which is synthetic DNA made for purpose of this study) is at 85°C. The melt curve was continuously detected with serially diluted RIME gBlock DNA even when mixed with other gBlock DNA of *Cryptosporidium* spp. or *T. congolense* and *T. gondii*. Amongst the subgenus *Trypanozoon* species, only *T. equiperdum* which causes a disease called dourine in equines occurs in South Africa (Gizaw *et al*., 2017, Herbet *et al.,* 2017; Williamson & Catherine, 1986), this means that this assay RIME HRM-qPCR assay can be used in the country for detection of *T. equiperdum* infections. The assay can further be used for detection of *T. b. gambiense* and *T. b. rhodesiense* in countries where sleeping sickness (human African trypanosomiasis) exist. The RIME HRM-qPCR assay amplified 10 times serially diluted gBlock DNA from 10^{-1} down to 10^{-8} which shows the detection limit of the assay to be down to 1 ag/µl (atto gram per microliter) [see appendix 1 for SI Units] which is equivalent to less than 1 trypanosome cell/ml. This shows the high detection sensitivity of the RIME HRM-qPCR

primers designed in this study which is similar or higher compared to the sensitivity of other DNA amplification methods for trypanosomes such as PCR and LAMP (Njiru *et al*., 2005; Thekisoe *et al.,* 2007). The assay is sensitive enough to detect the trypanosome parasites even though the parasitaemia count is low.

The RIME HRM-qPCR assay was further tested for amplification of DNA in comparison with conventional PCR using the same RIME gene primers from field derived samples with DNA extracted from cattle and tsetse flies. The RIME conventional PCR positively detected DNA of trypanosome in 4/10 (57.14%) and 6/10 (60%) of cattle and tsetse fly samples respectively. The RIME HRM-qPCR positively detected trypanosome DNA in 6/7 (85.71%) in the cattle samples and 8/10 (80%) in the tsetse fly samples. Although tested on few cattle and tsetse fly samples from animal trypanosome and tsetse area, the data indicates that the RIME HRM-qPCR has superior detection efficiency that RIME conventional PCR.

5.1.1. b) *T. congolense*

The causal agent of animal trypanosomiasis in South Africa is *T. congolense* in the KwaZulu-Natal province with evidence from studies of Motloang *et al.* (2014) *T. congolense* infections in cattle and tsetse flies collected in north-eastern KwaZulu-Natal, respectively.

The ribosomal P0 (rP0) gene sequence which was used by Kuboki *et al*. (2003) to develop a *T. congolense* LAMP assay was downloaded and used in this study. The *T. congolense* rP0 gene sequences were used on BLASTn for search of homologous gene sequences on the NCBI database. As a result, there were matches with rP0 genes expressed by *T. cruzi, T. b. brucei*, *T. b. gambiense* and *T. evansi*. Results of the sequence alignment has shown that rP0 gene is conserved amongst trypanosomes but has a lot of SNPs (Figure 4.2). This makes the rP0 gene a good candidate for the development of a species-specific diagnostic assay of the trypanosome species. In this study the rP0 primers were designed for specific amplification of *T. congolense* which is highly prevalent in the north-eastern parts of KwaZulu-Natal province of South Africa as well as the rest of tsetse infested countries of the African continent (Ntantiso *et al*., 2014; Masiga *et al*., 1996; Motloang *et al.*, 2014; Woolhouse *et al.,* 1993).

Optimization of the rP0 HRM-qPCR assay with gBlocks of rP0 gene of *T. congolense, T. cruzi, T. b. brucei* and *T. b. gambiense* showed the melt curve peaks ranging from 82 - 84°C with majority (15 out 20 reactions) being 83°C. This rP0 HRM-qPCR assay can be used to detect *T. congolense* in South Africa since *T. b. brucei, T. evansi* and *T. cruzi* do not occur in the country. The assay can further be explored for use in other African and Asian regions where *T. b. brucei* and *T. evansi* occur such as east Africa, central Africa and India (Brun *et al*., 2010; Elamin *et al*.,1998; Joshi *et al*., 2005). Moreover, the rP0 HRM-qPCR assay can also be assessed for detection of *T. cruzi* infection in South American countries where Chagas disease is prevalent (Schmunis, & Yadon,

2010). Figure 4.19 indicate the detection limit is only till 1 fg/µl that is a standard amplification plot for this study.

The rP0 HRM-qPCR assay amplified 10 times serially diluted gBlock DNA of *T. congolense* from 10^{-1} down to 10^{-8} which shows the detection limit of the assay to be down to 1 ag/ul (atto gram per microliter) which is equivalent to less than 1 trypanosome cell/ml. This shows the high detection sensitivity of the rP0 HRM-qPCR primers designed in this study which is similar or higher to reported sensitivity of other DNA amplification methods for *T. congolense* such as PCR and LAMP (Kuboki *et al*., 2003; Thekisoe *et al.,* 2007).

The rP0 conventional PCR for *T. congolense* detected 4/7 (57.14%) in the cattle samples and 5/10 (50%) in the tsetse fly samples PCR. Whilst rP0 HRM-qPCR assay detected 7/7 (100%) in the cattle samples and 7/10 (70%) in the tsetse fly samples. This further shows the superior detection performance of rP0 HRM-qPCR as compared to rP0 conventional PCR.

5.1.2. *Cryptosporidium* spp.

Cryptosporidium is the cause of increased neonatal diarrhoea in sheep and goats (de Graaf *et al.,* 1999). It has previously been stated that *Cryptosporidium* can infect birds and was first described by Tyzzer, (1929). It is a parasite prevalent in domestic and wild birds and have been detected in more than 30 bird species (Sréter & Varga, 2000). Furthermore, *Cryptosporidium* can also infect humans causing self-healing diarrhoea in immunocompetent patients but problematic in immunocompromised individuals (Hunter, & Nichols, 2002).

A successful LAMP assay was developed for the detection of *Cryptosporidium* species targeting the HSP70 gene by Bakheit *et al*. (2008). In this study, HSP70 gene sequences of *C. parvum, C. hominis, C. meleagridis and C. erinacei* were downloaded and aligned. The sequence alignment results showed that the HSP70 is highly conserved amongst these *Cryptosporidium* species with few SNPs existing (Figure 4.3.) This observation was an indication that the HSP70 gene is a good candidate for the development of a genus *Cryptosporidium* diagnostic assay. We therefore designed the HSP70 primers for amplification of *Cryptosporidium* species in South Africa. Bakheit *et al*. (2008) previously reported the presence of *Cryptosporidium* spp.

The optimization experiments of HSP70 HRM-qPCR consistently indicated the melt curve peak temperature to be 76°C for positive amplification of *Cryptosporidium* gBlock DNA which included *C. parvum, C. hominis, C. meleagridis and C. erinacei*. The melt curve was continuously detected with serially diluted HSP70 gBlock DNA. This HSP70 HRM-qPCR can be used for detection of *Cryptosporidium* spp. DNA worldwide in both animals and humans as *Cryptosporidium* is known to cause cosmopolitan infections distributed all over the world (Roberts *et al*., 2013). The HSP70 HRM-qPCR assay amplified 10 times serially diluted gBlock DNA of *Cryptosporidium* from 10^{-1} down to 10^{-8} (Figure 4.20) which shows the detection limit of the assay to be down to 1 ag/ul (atto

gram per microliter) and is equivalent to less than oocyst cell/ml. This shows the high detection sensitivity of the HSP70 HRM-qPCR primers designed in this study which is similar or higher to reported sensitivity of other DNA amplification methods for *Cryptosporidium* such as PCR and LAMP (Karanis *et al*., 2007; Bakheit *et al*., 2008). The HSP70 HRM-qPCR detected 5/10 (50%) of *Cryptosporidium* spp. infections from sheep samples and none from wild bird samples. Conventional PCR did not detect *Cryptosporidium* spp. infections from wild birds and sheep samples.

5.1.3. *Toxoplasma gondii*

This confirms the study done by Berger-Schoch *et al.* (2011) that *Toxoplasma gondii* infections are prevalent in sheep, pigs and cattle. There is a lot unknown about the prevalence of *T. gondii* in birds but due to birds feeding manner, birds can be environmental indicators of contamination (Bartova *et al*., 2009; Shaapan *et al*., 2011). The prevalence can either be detected serologically or from cysts in tissues of hosts (Literák & Hejlíček, 1993). The detection limit for *T. gondii* varies depending on the sensitivity assay being used (Shaapan *et al*., 2008).

Sequence alignment showed that a large region is conserved amongst *T. gondii* isolates with no single nucleotide polymorphisms thus making the B1 gene a good candidate for the development of a diagnostic assay. The optimization experiments of B1 HRM-qPCR consistently indicated the melt curve peak temperature to be 81°C for positive amplification of *T. gondii* gBlock DNA. The melt curve was continuously detected with serially diluted B1 gBlock DNA. These B1 HRM-qPCR primers can be used for detection of *T. gondii* DNA worldwide in both animals and humans as *T. gondii* is known to cause cosmopolitan infections distributed all over the world (Ramzan *et al*., 2009). The B1 HRM-qPCR assay amplified 10 times serially diluted gBlock DNA of *T. gondii* from 10^{-1} down to 10^{-8} (Figure 4.21) which shows the detection limit of the assay to be down to 1 ag/ul (atto gram per microliter) which is equivalent to less than oocyst cell/ml. This detection sensitivity is similar to other DNA based assays previously reported for detection of *T. gondii* such as LAMP (Zhang *et al.,* 2009). The assay is sensitive enough to detect the parasite even though the parasitic count is low.

For B1 HRM-qPCR detected 4/10 (40%) and 0% of *T. gondii* infections from sheep and wild bird samples, respectively. No DNA was detected with conventional PCR from wild birds and sheep and this could attribute to the low parasitaemia, particularly from sheep samples which were previously reported to be positive for *T. gondii* infections by conventional PCR and sequencing (Mofokeng, 2018). This data shows superior detection sensitivity of B1 HRM-qPCR as compared to conventional PCR.

5.2. Conclusion

In this study, HRM-qPCR assays were designed targeting three genes (RIME, rP0 and B1) for the detection of subgenus *Trypanozoon* trypanosomes, *T. congolense*, *Cryptosporidium* spp. and *T. gondii* respectively. The newly developed HRM-qPCR assays specifically amplified DNA of their respective target parasites even though it was prepared mixed with other species whereby each assay has its specific melt peak temperature. The assays have displayed good detection limit which can detect DNA equivalent to less than 1 cell or oocyst of target parasite. Furthermore, these RIME, rP0, HSP70 and B1 HRM-qPCR assays have shown the ability to detect DNA of their respective parasites from field derived samples with higher detection sensitivity than conventional PCR. This study has pioneered the development of HRM-qPCR for detection of *Trypanosoma* spp., *Cryptosporidium* and *T. gondii*. With further standardisation and validation experiments, these assays can be adopted for use in reference laboratories and be developed into commercial kits.

5.3. Recommendations

- Standardisation experiments should be conducted for each assay.
- Multiplex HRM-qPCR assays should also be experimented with a combination of these primers in one reaction mixture, using the same target genes.
- Further studies covering a larger screening of field samples should be conducted.

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ANNEXURES 1

deci	d	$1000^{-1/3}$	10^{-1}	0.1	tenth	1795
centi	c	$1000^{-2/3}$	10^{-2}	0.01	hundredth	1795
milli	m	1000^{-1}	10^{-3}	0.001	thousandth	1795
micro	μ	1000^{-2}	10^{-6}	0.000 001	millionth	1873
nano	n	1000^{-3}	10^{-9}	0.000 000 001	billionth / milliardth	1960
pico	p	1000^{-4}	10^{-12}	0.000 000 000 001	trillionth / billionth	1960
femto	f	1000^{-5}	10^{-15}	0.000 000 000 000 001	quadrillionth / billiardth	1964
atto	a	1000^{-6}	10^{-18}	0.000 000 000 000 000 001	quintillionth / trillionth	1964
zepto	z	1000^{-7}	10^{-21}	0.000 000 000 000 000 000 001	sextillionth / trilliardth	1991
yocto	y	1000^{-8}	10^{-24}	0.000 000 000 000 000 000 000 001	septillionth / quadrillionth	1991

Figure: SI unit table illustration (Source: Wikipedia).