Molecular and parasitological survey of trypanosomosis in KwaZulu-Natal and the development of cathepsin L-like real-time PCR assays for parasite discrimination

by

Samantha Mnkandla

Submitted in fulfilment of the requirements for the degree
Master of Science (Veterinary Science Tropical Diseases)

in the

Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

November 2020

© University of Pretoria

Declaration

I hereby declare that this dissertation, which I am submitting for a Master of Science degree at the University of Pretoria, is my own work, and has not previously been submitted by me for any degree or review at any other institution.

.... 17/03/21 ....

Samantha Mnkandla **Date**

© University of Pretoria

Acknowledgements

I wish to express my sincere gratitude and appreciation to my supervisors Prof. Luis Neves and Dr. Raksha Bhoora for giving me this opportunity to study at the University of Pretoria. I would like to thank them for their invaluable assistance and expertise throughout this degree, and their support and encouragement.

I would like to express my gratitude to Dr. Mbizeni for assisting in acquiring DAFF section 20 approval, mapping out the field sampling points and arranging for sample collection. Many thanks to Daniel Marumo for his assistance with the collection of blood samples and for his support and guidance. I would like to acknowledge to Ms Ilse Vorster for her assistance with laboratory training and technical inquiries.

I would like to thank the the Institute of Tropical Medicine in Antwerp and the Belgian Directorate-General for Development Co-operation Framework Agreement (FA4 DGD-ITM 2017-2021) that was awarded to the Department of Veterinary Tropical Diseases at the University of Pretoria, which funded this project. Many thanks to the Agricultural Research Council-Onderstepoort Veterinary Research for accommodating me and allowing me to use their facilities for processing my samples. I would like to thank the postgraduate students and the academic staff in Department of Veterinary Tropical Diseases for all their assistance and support throughout this study.

I would also like to thank my family and friends for their encouragement and support. Lastly, but most importantly, I would like to thank God for orchestrating this entire journey, glory be to Him.

© University of Pretoria

Table of Contents

Declaration .... **i**

Acknowledgements .... **ii**

Table of Contents .... **iii**

List of Figures .... **v**

List of Tables .... **vi**

List of Abbreviations .... **vii**

STUDY SUMMARY .... **ix**

CHAPTER 1 .... **1**

GENERAL INTRODUCTION 1

1.1 Introduction 1

1.2 Trypanosomes 1

1.3 Clinical signs and pathogenesis of African animal trypanosomosis 2

1.4 Classification of trypanosomes 4

1.5 *Trypanosoma* life cycle 6

1.6 Transmission 8

1.7 Tsetse flies 9

1.8 Trypanosomosis in Africa 10

1.9 Control 12

1.10 Diagnosis of Trypanosomes 14

1.11 Motivation 19

1.12 Aim and Objectives 20

CHAPTER 2 .... **21**

MATERIALS AND METHODS 21

2.1 Study area 21

2.2 Sampling 21

2.3 Molecular analysis 24

© University of Pretoria

2.4 Development of cathepsin L-like (CATL) real-time PCR assay 26
2.5 Statistical analyses 32
CHAPTER 3 33
RESULTS 33
3.1 Parasitological detection of trypanosomes 33
3.2 Molecular analysis 36
3.3 Development of the CATL qPCR assays 37
3.4 Comparing diagnostics techniques for the detection of trypanosomes 44
CHAPTER 4 46
DISCUSSION 46
4.1 Trypanosomes in cattle 46
4.2 Development of the CATL qPCR assays 50
4.3 Detection of trypanosomes in goats 53
CHAPTER 5 56
CONCLUSIONS 56
REFERENCES 58
APPENDICES 76

© University of Pretoria

List of Figures

Figure 1: Deteriorating body condition of a cow affected by AAT. 3

Figure 2: Classification of *Trypanosoma* genus and subgenus level (Stevens and Brisse, 2004). 4

Figure 3: The life cycle of *T. brucei* (Dyer *et al.*, 2013). 8

Figure 4: Ribosomal RNA gene structure (Cox *et al.*, 2005). 16

Figure 5: Map of the northern KwaZulu-Natal districts in South Africa. 22

Figure 6: The pJET1.2/blunt vector map. 29

Figure 7: Bar chart showing the percentage of anaemic and non-anaemic goats' samples at each diptank (n=128). 35

Figure 8: Agarose gel showing bands obtained from ITS PCR amplification using primers ITS1 CF and ITS1 BR. 36

Figure 9: Nucleotide sequence alignment of the target region of the *T. congolense* CATL-specific qPCR assay in the CATL gene of published *T. congolense* sequences. 38

Figure 10: Nucleotide sequence alignment of the target region of the *T. theileri* CATL-specific qPCR assay in the CATL gene of published *T. theileri* sequences. 39

Figure 11: Nucleotide sequence alignment of the target region of the *T. brucei* CATL-specific qPCR assay in the CATL gene of published *T. brucei* sequences. 40

Figure 12: PCR analysis of the recombinant clones using the *Trypanosoma* species-specific CATL-forward and genus-specific CATL-reverse primers designed for the qPCR assays... 41

Figure 13: Linear regression for the quantification of A) *T. congolense* B) *T. brucei* C) *T. theileri* parasite DNA. 42

© University of Pretoria

List of Tables

Table 1: Oligonucleotide primers based on the ITS1 gene for detecting and distinguishing between *Trypanosoma* species (McLaughlin *et al.*, 1996; Njiru *et al.*, 2004a). 17

Table 2: Sampling Sites in NKZN, (Ntantiso, 2012)... 21

Table 3: Universal primers and genus-specific ITS1 real-time probes for detecting African animal trypanosomosis (AAT) (Silbermayr *et al.*, 2013).. 26

Table 4: Primers and probes designed to amplify *Trypanosoma* CATL-like gene................ 27

Table 5: Trypanosome infection rates, HA-PCV and HAA in cattle at the three diptanks..... 34

Table 6: Average PCV and anaemia observed at the three diptanks in infected and non-infected groups of cattle. 35

Table 7: *Trypanosoma* CATL qPCR assays specificity.. 43

Table 8: Comparison of diagnostic techniques used in detecting trypanosomes. 45

© University of Pretoria

List of Abbreviations

AAT	African animal trypanosomosis
AC	Accession number
ANOVA	Analysis of variance
ARC	Agricultural Research Council
BCT	Buffy coat technique
BLAST	Basic Local Alignment Search Tool
bp	base pair
CATL	Cathepsin L
CP	Cysteine protease
Ct	Cycle threshold
DA	Diminazene aceturate
DAFF	Department of Agriculture, Forestry and Fisheries
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
DVTD	Department of Veterinary Tropical Diseases
E	Efficiency
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
gGAPDH	glycosomal glyceraldehyde-3-phosphate dehydrogenase
GPI-PLC	glycosyl-phosphatidylinositol-specific phospholipase C
HAA	Herd average anaemia
HA-PCV	Herd average packed cell volume
IDT	Integrated DNA Technologies, Inc.
ITS	Internal transcribed spacer
KZN	KwaZulu-Natal
LB	Luria Bertani
MGB	Minor groove binder
ml	millilitre
ng	nanograms
NKZN	Northern KwaZulu-Natal
OVR	Onderstepoort Veterinary Research

© University of Pretoria

PCR	Polymerase chain reaction
PCV	Packed cell volume
pmol/μL	picomoles per microlitre
qPCR	Quantitative real-time polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RoTat	Rode *Trypanozoon* antigen type
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
s.l	*senso lato*
SIT	Sterile insect technique
SOC	Super Optimal broth with Catabolite repression
SPSS	Statistical Package for Social Sciences
SSU	Small subunit
TAE	Tris-acetate EDTA
Tris-HCl	Tris-Hydrochloric acid
UK	United Kingdom
UP	University of Pretoria
USA	United States of America
UV	Ultraviolet
VSG	Variant surface glycoprotein
μg/mL	micrograms per millilitre
μl	microlitre
μM	micromolar

© University of Pretoria

STUDY SUMMARY

Molecular and parasitological survey of trypanosomosis in KwaZulu-Natal and the development of cathepsin L-like real-time PCR assays for parasite discrimination

by

Samantha Mnkandla (18392262)

Supervisor	: Prof. Luis Neves
Co-supervisor	: Dr. Raksha Bhoora
Degree	: MSc Veterinary Science Tropical Diseases
Department	: Veterinary Tropical Diseases, Faculty of Veterinary Science

African animal trypanosomosis (AAT), is an infectious parasitic disease of wildlife and livestock caused by multiple species and strains of *Trypanosoma*. In South Africa, it is restricted to northern KwaZulu-Natal (NKZN) and caused by *Trypanosoma congolense* and *Trypanosoma vivax*. A cross sectional study was conducted to determine AAT prevalence in 384 goat samples and identify trypanosome species circulating in 60 cattle at diptanks that are on the interface with Hluhluwe-uMfolozi game reserve in NKZN. Both cattle and goat samples were analysed using the buffy coat technique (BCT) and a polymerase chain reaction (PCR) assay targeting the internal transcribed spacer 1 (ITS) region. Cattle samples were further analysed using an ITS quantitative real-time PCR (qPCR) assay designed for the detection of *T. congolense*, *T. vivax* and *Trypanosoma brucei*. None of the goat samples tested positive for *Trypanosoma* infections. The ITS qPCR assay detected *Trypanosoma* DNA in 30% of the cattle samples, while only 8.3% were positive with the ITS PCR and 11.7% were positive using BCT. Quantitative real-time PCR assays were designed to amplify a 98 base pairs (bp), 137 bp and 116 bp fragment of the cathepsin L-like (CATL) gene from *T. brucei*, *Trypanosoma theileri* and *T. congolense*, respectively. Each assay was shown to be efficient (>94%) and specific (10^9 to $10^2/10^1$ copies/reaction) in the detection of *Trypanosoma* species. The diagnostic efficiency of the CATL qPCR assays was further tested using 60 cattle and 39 goats' blood samples collected at three diptanks in NKZN. The CATL qPCR assays detected *T. congolense* and *T. theileri* infections in 33.3% of the cattle samples. The CATL qPCR

© University of Pretoria

assays also detected *T. congolense* infections in goats (23.1%) that were neither detected by BCT nor the ITS PCR. The CATL qPCR assays provide an additional, sensitive and specific tool for *Trypanosoma* diagnostics. The presence of trypanosomes in goats suggests they might be potential reservoirs of infections to other livestock.

Keywords: African animal trypanosomosis, cathepsin L, real-time PCR, ITS PCR, *Trypanosoma*, buffy coat technique.

© University of Pretoria

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Livestock are a major contributor to the economy of developing countries but despite this, diseases such as trypanosomosis continue to be a constraint to the productivity of the livestock-sector in these regions (Uilenberg, 1998; Alsan, 2015). African animal trypanosomosis (AAT) is a debilitating disease of livestock caused by extracellular flagellate protozoan parasites belonging to the family Trypanosomatidae (Desquesnes, 2004). Three species of *Trypanosoma* endemic to Africa are *Trypanosoma vivax*, *Trypanosoma congolense* and *Trypanosoma brucei senso lato* (*s.l*) (Courtin *et al.*, 2008).

Trypanosomosis has been reported globally as one of the top ten cattle diseases that affect poor communities (Perry *et al.*, 2002). It affects livestock that are involved in farming and transportation (Tchamdja *et al.*, 2017), restricting rural agricultural production, crop-livestock agricultural practices, as well as livestock health (Walshe *et al.*, 2009). The major impacts of the disease on livestock (cattle, goats and sheep) is mortality and reduced productivity, in terms of, poor growth and calving rates, decreased milk production, and general reduction in work productivity of draft animals (Shaw *et al.*, 2014). African animal trypanosomosis is endemic to 37 of the 54 African countries, including South Africa (Courtin *et al.*, 2008). Over 50 million cattle in Africa are at risk of getting trypanosome infections and approximately 3 million cattle are killed by AAT each year (Eyford *et al.*, 2011). In a study done in East Africa by Shaw *et al.* (2014), annual losses due to trypanosomiasis are estimated to reach approximately US$4.5 billion while intervention against AAT could potentially benefit the economy by US$2.5 billion.

1.2 Trypanosomes

Although David Bruce (1855-1931) was the first to identify trypanosomes, the impact of the disease on animals in Zululand and across Africa was recognized long before by locals and missionaries such as David Livingstone (1813-1875) (Connor, 1994; Cook, 1994). David Bruce identified the cause of the disease to be a “Hematozoa” (later renamed to *Trypanosoma brucei*), transmitted by tsetse flies (*Glossina spp*) (Cook, 1994).

© University of Pretoria

Trypanosomes are haemoflagellated, parasitic protozoans that belong to the genus *Trypanosoma* (Kinetoplastida: Trypanosomatidae) (Desquesnes, 2004). These parasites cause infections in both animals and humans. *Trypanosoma* infections in animals can either be chronic or acute. *Trypanosoma vivax* infections are often acute compared to *T. congolense* infections that are often chronic (Firesbhat and Desalegn, 2015). Trypanosomes cause single infections, however, mixed infections by different trypanosome species and subspecies are very common in livestock (Murray and Trail, 1987; Mamabolo *et al.*, 2009; Silbermayr *et al.*, 2013). The distribution of the major trypanosomes affecting mammals, except *T. vivax*, across Africa is dependent on the distribution of tsetse flies in the tsetse belt (Connor and Van den Bossche, 2004). *Trypanosoma vivax*, *Trypanosoma evansi*, *Trypanosoma (Megatrypanum)*, *Trypanosoma cruzi* and *Trypanosoma equiperdum* occur outside the tsetse belt of Africa (Rodrigues *et al.*, 2003; Moncayo and Silveira, 2007; Rassi *et al.*, 2012; Eberhardt *et al.*, 2014; Xavier *et al.*, 2014; Bono Battistoni *et al.*, 2016), due to their adaptation to non-tsetse fly method of transmission (Rodrigues *et al.*, 2005; Gibson *et al.*, 2017).

1.3 Clinical signs and pathogenesis of African animal trypanosomosis

Clinical signs of infected cattle are determined by the pathogenicity of the *Trypanosoma* species, cattle breed type, exposure duration and the health status of the cattle (Chamond *et al.*, 2010; Firesbhat and Desalegn, 2015). Anaemia has been determined as the major symptom and can become chronic if left untreated (Van den Bossche and Rowland, 2001; Eyford *et al.*, 2011). Other signs of AAT include loss of body condition (Figure 1), abortion and still births in pregnant hosts, infertility, paralysis and neuroendocrine dysfunctions, edema, enlarged lymph nodes and loss of appetite (Darji *et al.*, 1992; Firesbhat and Desalegn, 2015; Steverding, 2008; Moti *et al.*, 2014; *Yaro et al.*, 2016) and death if not treated.

When trypanosomes are released into the blood circulation system and the lymphatic system of the host they result in hyperthermia and some of the abovementioned clinical signs (*Yaro et al.*, 2016). Hyperthermia comes as a result of the host body defence system in response to the expressed *Trypanosoma* surface proteins. The mammalian host makes antibodies specific to proteins being expressed by the trypanosomes and some trypanosomes replace their surface proteins with new proteins. The trypanosomes that did not replace their surface proteins are destroyed by the antibodies. The surviving trypanosomes replicate, induce parasitaemia and subsequently, induce hyperthermia (Uilenberg, 1998; Matthews *et al.*, 2015). The host responds by making specific antibodies to the new surface proteins being expressed, and some trypanosomes replace their surface coat proteins before the antibodies destroy them, and the cycle repeats. Trypanosomes are capable of making infinite antigenic

© University of Pretoria

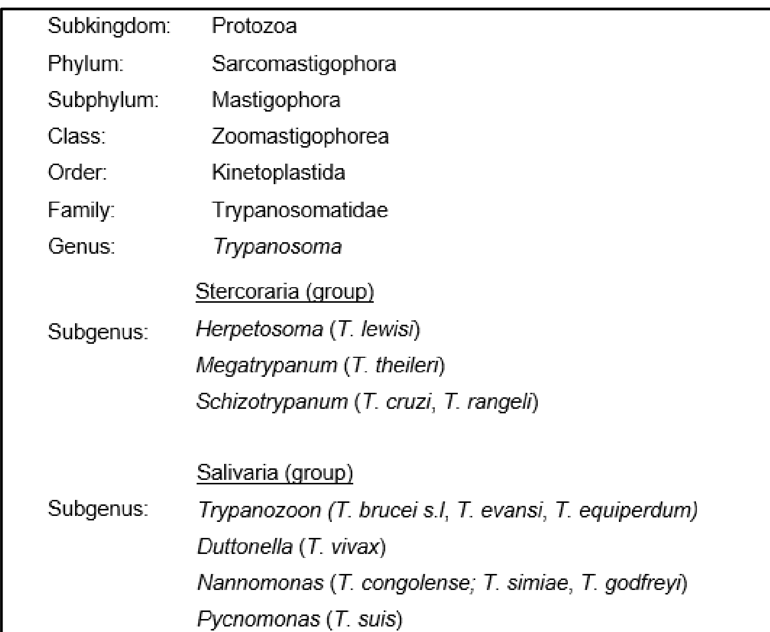
variants and this cycle goes on for a very long time until the host dies, self-cures, or is treated (Uilenberg, 1998; Matthews *et al*., 2015).

Figure 1: Deteriorating body condition of a cow affected by AAT.

© University of Pretoria

1.4 Classification of trypanosomes

Based on their mode of transmission trypanosomes are classified as either salivarian or stercorarian (Hoare, 1966) (Figure 2). Most stercorarian trypanosomes are non-pathogenic and most salivarian trypanosomes are pathogenic (Hoare, 1972; Firesbhat and Desalegn, 2015). Salivarian trypanosomes develop in the alimentary canal of the insect, develop into infectious forms in the salivary glands and proboscis, and are transmitted by mouthparts of the insect vectors (Holmes, 2013; Firesbhat and Desalegn, 2015). The subgenus groups of salivarian trypanosomes are *Trypanozoon*, *Duttonella*, *Pycnomonas* and *Nannomonas*. Vectors that transmit salivarian trypanosomes include tsetse flies (*Glossina spp*) and biting flies (*Tabanus*, *Stomoxys* and *Lyperosia spp*) (Mihok *et al.*, 1995; Walshe *et al.*, 2009; Desquesnes *et al.*, 2013). *Trypanosoma congolense* is the most economically significant of the *Nannomonas* salivarian trypanosome species (Walshe *et al.*, 2009). Trypanosomes of the stercorarian group develop in the intestinal tract of the insect vector and are transmitted through the insect faecal matter. Subgenus groups of stercorarian trypanosomes are *Herpetosoma*, *Schizotrypanum* and *Megatrypanum* (Firesbhat and Desalegn, 2015). Vectors that transmit these trypanosomes include reduvids, tabanids, ticks and leeches (Lent and Wygodzinsky, 1979; Walshe *et al.*, 2009).



Subkingdom:	Protozoa
Phylum:	Sarcomastigophora
Subphylum:	Mastigophora
Class:	Zoomastigophorea
Order:	Kinetoplastida
Family:	Trypanosomatidae
Genus:	*Trypanosoma*
	Stercoraria (group)
Subgenus:	*Herpetosoma* (*T. lewisi*)
	Megatrypanum (*T. theileri*)
	Schizotrypanum (*T. cruzi*, *T. rangeli*)
	Salivaria (group)
Subgenus:	*Trypanozoon (T. brucei s.l, T. evansi, T. equiperdum)*
	Duttonella (*T. vivax*)
	Nannomonas (*T. congolense; T. simiae*, *T. godfreyi*)
	Pycnomonas (*T. suis*)

Figure 2: Classification of *Trypanosoma* genus and subgenus level (Stevens and Brisse, 2004).

© University of Pretoria

1.4.1 Trypanosome species

1.4.1.1 *Trypanosoma brucei*

Trypanosoma brucei s.l are found across sub-Saharan Africa and infect humans, livestock and wildlife. This species represented by three sub-species; *Trypanosoma brucei brucei*, which only infects livestock, *Trypanosoma brucei rhodesiense*, a livestock and human parasite and *Trypanosoma brucei gambiense*, which primarily infects humans, but can infect other mammals (Baker, 1995; Walshe *et al.*, 2009). *Trypanosoma brucei* is the most extensively studied trypanosome species, with the most understood life cycle due to its procyclic life cycle stage that can easily be cultured on liquid media, *in vitro* (Matthews, 2015; Gibson *et al.*, 2017). *Trypanosoma brucei* can also multiply in great numbers inside rodents and this has enabled many breakthroughs in the study of trypanosomes such as the life cycle, antigenic variation mechanism and the mitochondrial structure of trypanosomes (Gibson *et al.*, 2017).

1.4.1.2 *Trypanosoma congolense*

Three genetically distinct types of *T. congolense* have been identified, namely, *T. congolense*-Forest-type (low-pathogenic), *T. congolense* Savannah-type (virulent) and *T. congolense* Kilifi-type (non-pathogenic) (Majiwa *et al.*, 1985; Majiwa *et al.*, 1993; Bengaly *et al.*, 2002). *Trypanosoma congolense* Savannah-type is found across Sub-Saharan Africa and infects a broad range of animal hosts (Bengaly *et al.*, 2002; Auty *et al.*, 2015). *Trypanosoma congolense* Kilifi-type has been reported in East and Southern Africa to infect cattle, sheep and goats (Majiwa *et al.*, 1985; Masiga *et al.*, 1996; Njiru *et al.*, 2004b; Mamabolo *et al.*, 2009; Simo *et al.*, 2012; Simo *et al.*, 2013). Although cases of *Trypanosoma congolense* Forest-type have been reported in East Africa and Zambia, it is mainly found in West and Central Africa, and has been isolated in cattle, dogs, pigs and goats (Auty *et al.,* 2015).

Mixed infection by the *T. congolense* subgroups is common. Co-infections of all subgroups have been reported in Kenya, Zambia and Tanzania (Masiga *et al.*, 1996; Njiru *et al.*, 2004b; Seck *et al.*, 2010; Simo *et al.*, 2012; Simo *et al.*, 2013). Mixed trypanosome infections caused by *T. congolense* Kilifi-type and Savannah-type, have also been reported in northern KwaZulu-Natal (NKZN), in areas around the Hluhluwe-uMfolozi game reserve (Mamabolo *et al.*, 2009; Gillingwater *et al.*, 2010).

© University of Pretoria

1.4.1.3 *Trypanosoma vivax*

Trypanosoma vivax is mainly found across sub-Saharan Africa with cases reported in South America (Auty *et al.*, 2015). It is transmitted either cyclically by tsetse flies or mechanically by biting flies (*Stomoxys* and *Tabanids*) in tsetse free regions (Raymond and Rousseau, 1987; Jones and Davila, 2001; Desquesnes and Dia, 2003; Auty *et al.*, 2015). The spread of *T. vivax* to tsetse free areas, such as South America, is believed to be through importation of West African cattle (Gardiner and Mahmoud, 1992). *Trypanosoma vivax* can be further classified as West African group or East African group based on their molecular and morphological features (Hoare, 1972; Cortez *et al.*, 2006). The West African strain has been reported to be more virulent than the East African strain (Gardiner and Mahmoud, 1992).

1.4.1.4 *Trypanosoma theileri*

Trypanosoma theileri (*Megatrypanum*) is not pathogenic, compared to *T. congolense*, *T. brucei* and *T. vivax* (Desquesnes, 2004). However, *T. theileri* allows simultaneous infections with other parasites such as *Anaplasma, Babesia* and *Theileria*, combined with other factors such as poor nutrition and stress, can cause chronic infections (Doherty *et al.*, 1993; Seifi, 1995; Rodrigues *et al.*, 2010). Therefore, *T. theileri* is of concern because it can confuse parasitological diagnosis (Uilenberg, 1998). *Trypanosoma theileri* can be transmitted cyclically by tsetse flies or mechanically by tabanids or ticks. The mature bloodstream forms taken up by tabanids replicate and differentiate into epimastigotes, and then differentiate trypomastigotes, and finally into the infective metacyclic forms inside the insects' midgut (Hoare, 1972; Desquesnes, 2004). The metacyclic trypanosomes are passed out of the insect's body through faecal matter and are transmitted to hosts through wounds or ingestion by the mammal (Desquesnes, 2004).

1.5 *Trypanosoma* life cycle

Life cycle of trypanosomes occurs in both the host and the vector (Dyer *et al.*, 2013), and the life cycle in the vector takes up to 3 weeks (Uilenberg, 1998). Trypanosomes, before being transmitted by tsetse flies to host, undergo a developmental cycle that varies among species (Abdi *et al.*, 2017). When tsetse flies, of both sexes, feed on blood of infected animals, they take up trypanosomes with their meal. In the tsetse fly, trypanosomes undergo a developmental cycle and are then transmitted via saliva when the tsetse fly feeds on another host (Gibson *et al.*, 2017).

© University of Pretoria

During a bloodmeal, the tsetse fly pierces the mammalian host skin with its proboscis. Blood from the punctured blood vessel collects in the tissues and the tsetse fly stop coagulation by injecting its saliva. Metacyclics undergo development and proliferation at the site of infection. Inflammation or chancre may be detected in the skin of the mammal (Urquhart *et al.*, 1996; Holmes, 2013). Mature bloodstream forms are released, via lymph nodes and lymph vessels, into circulation (Holmes, 2013). Within the host, trypanosomes feed and digest protein, carbohydrates and fats taken across the outer membrane from the host (Uilenberg, 1998). When a tsetse fly ingests a blood meal carrying trypanosomes, the parasite replaces the existing variable surface glycoprotein (VSG) molecules with procyclins and the blood stream form differentiate to a procyclic form (Gibson and Bailey, 2003). As described by Gibson and Bailey, (2003), many tsetse flies can destroy trypanosomes during a process called self-cure and, for the first 3 days, the trypanosomes that survive are kept in the ingested blood meal.

After 3 days the trypanosomes can either replicate to establish an infection or they die. The *T. brucei* and *T. congolense* procyclics that manage to initiate an infection move to the midgut where replication occurs, while *T. vivax* procyclics skip the midgut and migrate to replicate in the proboscis (Walshe *et al.*, 2009; Dyer *et al.*, 2013; Holmes, 2013). The *T. brucei* and *T. congolense* procyclics migrate to the proventriculus where the trypanosomes gather and differentiate to mesocyclic form and consequently, trypomastigotes (Walshe *et al.*, 2009; Gibson *et al.*, 2017) (Figure 3). However, the *T. brucei* trypomastigotes migrate through the foregut to the salivary glands where they transform into the metacyclic forms, while the *T. congolense* trypomastigotes migrate from the midgut to the proboscis (Dyer *et al.*, 2013). In the proboscis, the *T. vivax* procyclics differentiate into trypomastigotes, and these *T. vivax* trypomastigotes and the *T. congolense* trypomastigotes differentiate into epimastigotes and subsequently, the metacyclic forms (Vickerman *et al.*, 1988; Holmes, 2013). All the metacyclic forms have VSG molecules found on the surface are expressed differently from bloodstream form VSGs (Walshe *et al.*, 2009; Gibson *et al.*, 2017). Each metacyclic parasite undergoes necessary changes that enables them to be transmitted and survive within the mammalian host. An infected fly will produce mature metacyclics for the rest of its life. Thus, infected tsetse flies are capable of infecting new hosts whenever they feed, and the flies are also vulnerable to infections by different trypanosome species causing mixed infections (Walshe *et al.*, 2009).

© University of Pretoria

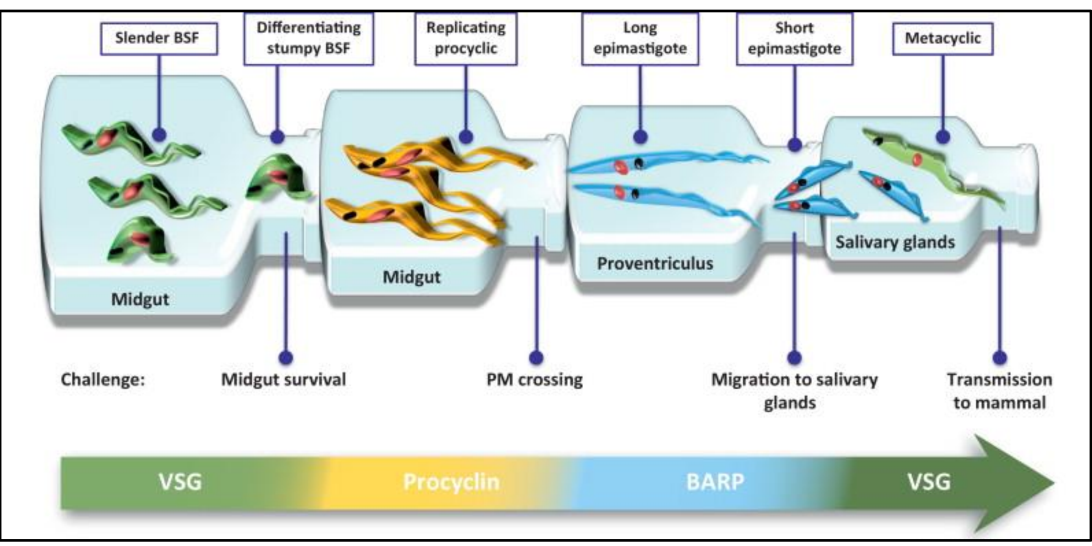


Figure 3: The life cycle of *T. brucei* (Dyer *et al.*, 2013).

1.6 Transmission

Transmission of trypanosomes can either be cyclical or mechanical. Trypanosomes such as *T. vivax* and *T. theileri* can be transmitted both cyclically and mechanically. During mechanical transmission biting insects transmit trypanosomes from an infected mammalian host to another host without going through any developmental stages within the insect (Uilenberg, 1998). For transmission to be effective, trypanosomes must be transmitted before the blood taken up dries. Mechanical transmission is dependent on the availability of the biting insect, the trypanosome species being transmitted and the number of hosts. Large biting insects such as tabanids are good mechanical vectors because they can carry more blood which consequently takes time to dry up (Uilenberg, 1998). Mechanical transmission has maintained trypanosomes outside the tsetse belt.

Male and female tsetse flies are of veterinary and medical importance because they cyclically transmit trypanosomes during a blood meal to both animals and humans (Krinsky, 2002; Walshe *et al.*, 2009; Abdi *et al.*, 2017). *Trypanosoma brucei*, *T. congolense*, *T. vivax* and *T. theileri* are cyclically transmitted. Tsetse flies infected with trypanosomes remain infective for a long period of time and can infect several mammalian hosts during feeding (Murray and Trail, 1987). Cyclical transmission of trypanosomes by tsetse flies is determined by the prevalence of trypanosomosis in the tsetse population (Abdi *et al.*, 2017). In South Africa,

© University of Pretoria

prevalence of trypanosomosis is higher during autumn months, when tsetse flies are in great numbers, and lower in the drier months (Gillingwater *et al.,* 2010).

1.7 Tsetse flies

Tsetse flies belong to the genus *Glossina* and are the major cyclical vectors that transmit trypanosomes (Abdi *et al.*, 2017). There are 23 species of tsetse flies that occur in 38 sub-Saharan African countries (Kristjanson *et al.*, 1999; Connor, 1994; Walshe *et al.*, 2009). From these, 8 - 10 species are of economic importance (Vreysen *et al.*, 2013). Tsetse fly species can further be divided into three subgenera, namely, *palpalis*, *fusca* and *morsitans* (Krinsky, 2002; Walshe *et al.*, 2009). The *mor*sitans group are good vectors of all *T. congolense* subtypes, but poor vectors of *T. b. gambiense*. These tsetse flies feed on wildlife and livestock. *Morsitans* occur in open land such as savanna woodlands and thickets. Species include *Glossina pallidipes* and *Glossina austeni* (Krinsky, 2002). The *palpalis* group are good vectors of *T. b. gambiense* (Walshe *et al.*, 2009). *Palpalis* are often found in vegetation around water bodies such as rivers, lakes and streams, and species found in the group include *Glossina fuscipes* (Walshe *et al.*, 2009; Krinsky, 2002). The *fusca* group are usually found in forested habitats including swamps and rain forests and include *Glossina brevipalpis* and *Glossina fusca*. With the exception of *G. brevipalpis*, tsetse flies in the *fusca* group are not of veterinary or medical importance (Walshe *et al.*, 2009; Krinsky, 2002).

Initially, four tsetse fly species (*Glossina morsitans morsitans*, *G. pallidipes, G. austeni* and *G. brevipalpis*) were recorded in South Africa (Du Toit, 1954; de Beer *et al.*, 2016). *Glossina morsitans morsitans* disappeared by 1897 during the rinderpest epidemic of 1896-1897 due to the reduction of hosts such as cattle and wildlife (de Beer *et al.*, 2016). Consequently, three *Glossina* species remained in NKZN. *Glossina pallidipes* remained the most abundant with cases of 8 million tsetse flies being caught by Harris traps in 1932 (Harris, 1932; de Beer *et al.*, 2016). This led to trypanosomosis outbreaks between 1942 and 1946 (Du Toit, 1954). To eradicate *G. pallidipes*, several methods were carried out between 1945 and 1952, including, aerial spraying of hexachlorobenzene and 1,1,1-trichloro-2,2-di (4-chlorophenyl) ethane (DDT), clearing vegetation, destruction of game animals and tsetse fly trapping by Harris traps. By 1953, *G. pallidipes* was completely eradicated from KZN (Du Toit, 1954).

Consequently, *G. brevipalpis* and *G. austeni* became the only two tsetse fly species left in South Africa. Although *G. brevipalpis* is mainly found in dense vegetation, Esterhuizen *et al.* (2005) also found it present in exotic plantations and open grasslands. *Glossina brevipalpis* has been reported to be the more abundant species than *G. austeni* (Bagnall, 1993; Kappmeier, 2000; Van den Bossche *et al.*, 2006). However, Motloang *et al.* (2012) showed

© University of Pretoria

that despite the low abundance of *G. austeni*, it has higher vector competence for *T. congolense* compared to *G. brevipalpis*.

1.8 Trypanosomosis in Africa

A meta-analysis study of trypanosomosis prevalence data, carried out by Ebhodaghe *et al.* (2018), from 19 sub-Saharan African countries gave an average prevalence of 15.10%. This study showed that AAT is highly prevalent in most of the endemic sub-Saharan African countries regardless of the control strategies adopted.

1.8.1 Trypanosomosis in cattle in South Africa

In South Africa, AAT is restricted to NKZN covering an area of 18 000km^2 that makes up part of the tsetse belt (Esterhuizen *et al.*, 2005). The area stretches from north of uMfolozi River to the southern Mozambique border (Sigauque *et al.*, 2000) and has over 300 000 head of cattle (Kappmeier *et al.*, 1998; Ntantiso *et al.*, 2014). African animal trypanosomosis is most prevalent in two municipalities, Big 5 Hlabisa and Mtubatuba that fall within the uMkhanyakude District (DC27). These two municipalities are of interest as they not only fall within the 18 000km^2 tsetse fly belt but are also on the interface with the Hluhluwe-uMfolozi game reserve where the dominant tsetse fly species in South Africa, *G. austeni* and *G. brevipalpis*, are most prevalent (Kappmeier Green, 2002; Ntantiso, 2012; de Beer *et al.*, 2016). Hlabisa Municipality covers an area of 3466km^2 and it is widely used for game activities, agriculture and local settlements, which are mostly rural settlements, while, Mtubatuba Municipality covers an area of 1970km^2 and is widely known for its wetlands and game reserves.

In 1990, an outbreak of AAT that occurred concurrently during a drought, killed around 10 000 cattle and was due to infection with both *T. congolense* and *T. vivax* (Bagnall, 1993; Kappmeier *et al.*, 1998; Emslie, 2004). Infected cattle were identified in 61 of the 132 diptanks surveyed and 116 000 cattle were treated with ethidium bromide (Kappmeier *et al.*, 1998). Additional control measures implemented, included changing the cattle-dipping regime from Amitraz (used for tick control) to pyrethroid cyhalothrin, which is effective against flies. The widespread occurrence of the disease, as noted by subsequent disease epidemiology and tsetse distribution surveys indicated that the role of *G. brevipalpis* and *G. austeni* in disease transmission had been severely underestimated (Bagnall, 1993; Kappmeier *et al.*, 1998; Emslie, 2004) and 16 years later, this was confirmed by the observed increased incidence of *T. congolense* infections at a single diptank at the edge of the Hluhluwe-uMfolozi game reserve (Van den Bossche *et al.*, 2006). A subsequent survey (2005-2008) targeting 10

© University of Pretoria

communal diptanks, confirmed that the incidence of *Trypanosoma congolense* had indeed increased over the years (Ntantiso *et al.*, 2014).

In a study done by Gillingwater *et al.* (2010), which used a *T. congolense* specific PCR, *T. congolense* Savannah-type was found to be the most dominant species (35.1% prevalence) circulating at four diptanks and a commercial farm in NKZN. Taioe, (2013) found an AAT prevalence of 23.4% in cattle around the uMkhanyakude district of KwaZulu-Natal, and none in goats and sheep. Only single infections of *T. congolense* and *T. theileri* were reported (Taioe, 2013). African animal trypanosomosis is currently an overlooked disease in South Africa with no control strategies put in place for the past 20 years (Ntantiso *et al.*, 2014; de Beer *et al.*, 2016; Latif *et al.*, 2019).

1.8.2 Trypanosomosis in goats

Small ruminants, such as goats, are very important in Sub-Saharan Africa as they provide a bulk of food resources for humans. Over 173 million goats in Africa, are found along the tsetse-belt and are at risk of contracting *Trypanosoma* infections (Gutierrez *et al.*, 2006). In the rural households of NKZN, goats not only provide meat and skins but also play an important role at cultural and social gatherings, and serve as an indication of wealth. Symptomatic infections with *Trypanosoma* rarely occur in goats and the disease is therefore considered to be of less significance. Studies have reported that small ruminants do not develop chronic *Trypanosoma* infections, even in tsetse infested areas (MacLennan, 1970; Stephen, 1970; Mahmoud and Elmalik, 1977). This may be related to the shorter generation time of small ruminants and greater natural selection for trypanotolerance in these species compared to cattle (Griffin, 1978; Oladele and Adenegan, 1998). However, AAT does affect small ruminant health and productivity (Griffin and Allonby, 1979; Hendy, 1988; Irungu *et al.*, 2002). African animal trypanosomosis research on goats could help improve productivity and food security especially in the tsetse infested areas.

A study was conducted by Bealby *et al.* (1996) to determine the effects of natural *Trypanosoma* infection on goats' health and productivity. The experiment ran over a period of four years in a high tsetse challenge area. The trypanosomosis prevalence for each year was dependent on the tsetse fly densities of that year, that is, the years that had high tsetse densities correlated with high mean trypanosomosis prevalence. Bealby *et al.* (1996) determined *T. congolense*, *T. brucei* and *T. vivax* to be causative species of AAT in the goats. *Trypanosoma vivax* and *T. congolense* were more prevalent than *T. brucei*, and *T. brucei* frequently appeared in mixed infections. These trypanosomes were transmitted to goats by *G. brevipalpis*, *G. pallidipes* and *G. m. morsitans*.

© University of Pretoria

The study highlighted the importance of trypanocidal treatment in small ruminants. Chemoprophylaxis protected goats had good health status compared to goats that did not receive any trypanocidal drug treatment. A high mortality rate of 42% was recorded in untreated goats during the first year of the experiment. Untreated infected goats compared to the treated goats, had lower packed cell volume (PCV) values. Untreated goats showed AAT clinical signs such as reduced body weight, anaemia, fever and death. Treated goats were more fertile, had higher body weights and better conception rates than untreated goats. Kids from untreated dams had lower birth weight than kids of treated dams.

The abovementioned research and other studies on goats experimentally infected with trypanosomes (Azab and Abdel-Maksoud, 1999; Tambuwal *et al.*, 2002), have shown that AAT infections in goats should not be overlooked as the diseases is associated with still births, abortions, low conception rates and reduced fertility, which ultimately reduces herd growth, milk production as well as the quality and quantity of skins and meat.

1.9 Control

Control of trypanosomosis can be achieved by either controlling the vector or controlling the trypanosomes.

1.9.1 Vector control

Vector control is carried out using traps, targets, netting, use of insecticides and the sterile insect technique (SIT) (Leak, 1999; Esterhuizen and Van den Bossche, 2006). Bush clearing was an ecological control method that stripped habitats for the savannah tsetse flies. However, bush clearing and killing of game animals previously used as vector control strategies were deemed inappropriate and have since been banned (Schofield and Maudlin, 2001; Torr and Vale, 2015). Aerial spraying with insecticides such as Dichlorodiphenyltrichloroethane (DDT) was largely criticized because of its potential damage to non-targeted organisms (Schofield and Maudlin, 2001). Traps, introduced around 1910, continue to be used as an effective control strategy for tsetse flies. Baited traps treated with insecticides attract female tsetse flies and suppress their population (Vale, 1993). Cattle dipping regime initially used to control ticks changed from using amitraz (insecticide used for tick control) to cyhalothrin, a pyrethroid capable of controlling both ticks and tsetse flies, has been widely used in NKZN (Bagnall, 1993; Brown, 2008).

Sterile insect technique is another method of tsetse fly control, used as part of an integrated vector control strategy. It is an environmentally friendly strategy that is used after tsetse

© University of Pretoria

population has been suppressed by insecticides, traps and/or targets (Dyck *et al.*, 2005; Abd-Alla *et al.*, 2013). It involves treating artificially reared male tsetse flies with mutagenic agents that induce sterility. The sterile males are then released and compete with wild males to mate with wild female tsetse flies. When these sterile male tsetse flies mate with wild female tsetse flies, they do not reproduce. Successful SIT control entails continuous release of male tsetse flies and in turn the tsetse population significantly decreases (Uilenberg, 1998). This strategy was implemented in Unguja, Zanzibar and resulted in the successful eradication of *G. austeni* (Vreysen *et al.*, 2000). Despite being a good vector control method, SIT, is very expensive and laborious (Uilenberg, 1998).

1.9.2 Chemotherapy

Chemotherapy and chemoprophylaxis are the major means of combatting the disease ensuring animal health and production in developing countries. There are currently six licensed compounds available for the treatment of trypanosomiasis (Giordani *et al.*, 2016), of these, diminazene aceturate (DA, Berenil®), which has curative properties (Chitanga *et al.*, 2011), is commonly used in South Africa. Extensive and incorrect utilization of these drugs has led to the emergence of resistant parasites in the field and there are increasing reports of multiple drug resistance (Delespaux *et al.*, 2008, Mamoudou *et al.*, 2008, Tekle *et al.*, 2018). However, DA-resistance mutation has also been observed in trypanosomes that have no history of exposure to drugs (Chitanga *et al.*, 2011). In South Africa, Berenil®, the trade name for diminazene aceturate, is widely used for the treatment of babesiosis and in clinical instances for African trypanosomiasis in livestock. Berenil® (DA) is easily assessable and the livestock owners can carry out treatment, themselves. The indiscriminate use of drugs has been implicated in the emergence of drug resistance, which becomes a serious problem in the control of trypanosomiasis, particularly for resource-poor farmers (Assefa and Shibeshi, 2018).

1.9.3 Vaccines

There are currently no vaccines to prevent trypanosomosis. Development of vaccines is difficult as most trypanosomes show antigenic variation (Gibson *et al.,* 2017). Trypanosomes have a wide array of variant surface glycoprotein (VSG) encoding sequences and recombination events ensures that these parasites generate an endless assemblage of VSGs (Walshe *et al.*, 2009; Firesbhat and Desalegn, 2015). This antigenic variation subsequently results in the evasion of the host immune response system by the parasite until the host succumbs to either reinfection by other trypanosome species with a different antigenic type or secondary infections or infections associated with complications (Firesbhat and Desalegn,

© University of Pretoria

2015). In the recent years, however, there has been interests in anti-disease strategies that reduce pathological factors, such as anaemia, instead of complete elimination of the parasite from the host (Baral, 2010).

1.10 Diagnosis of Trypanosomes

The application of appropriate control strategies requires proper understanding of the epidemiology of the disease and accurate diagnosis of trypanosomes. Field diagnosis of AAT usually involves identifying a herd with poor health status or an individual anaemic animal with poor body condition (Van den Bossche *et al.*, 2006). However, diagnosis of trypanosomosis should not solely be based on clinical signs as several other diseases show similar symptoms (Moti *et al.*, 2014). Some of the diagnostic methods include parasitological techniques and molecular assays.

1.10.1 Parasitological Techniques

Morphological identification of blood stream trypanosomes can be done by microscopy (Gibson *et al.*, 1999). Parasitological techniques, that include, stained blood smears and buffy coat technique (BCT), have good specificity but lack sensitivity under field conditions and are usually ineffective in cases with low parasitaemia (Moti *et al.*, 2014). These methods also require a considerable amount of time and effort (Cox *et al.*, 2005). Identification of trypanosomes within the vector is also difficult to do by microscopy as some of the trypanosome species are indistinguishable within the vectors (Gibson *et al.*, 1999). Despite these limitations, parasitological techniques are still preferred in sub-Saharan Africa as they are cost effective (Odongo *et al.*, 2016).

1.10.2 Molecular assays

Low levels of parasitaemia often pose a challenge in AAT prevalence studies and differentiating the trypanosome species is important in epidemiological studies and is usually achieved by use of molecular assays (Silbermayr *et al.*, 2013). Molecular diagnostic tools have been improved over the years making them more accurate, efficient, sensitive and faster (Moser *et al.*, 1989). Molecular diagnosis tools include, conventional polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP) and real-time PCR.

© University of Pretoria

1.10.2.1 Conventional polymerase chain reaction

One of the most specific and sensitive molecular diagnostic tools in both the vector and the host, is the PCR. Polymerase chain reaction is an efficient tool for use in prevalence studies of AAT as it detects trypanosome infections with more specificity (Gibson *et al.*, 1999). Despite PCRs being costly and time consuming and requiring technical expertise, they have significantly enhanced sensitivity and improved accuracy in diagnosis of trypanosome infection in contrast to parasitological techniques such as BCT and blood smears (Picozzi *et al.*, 2008).

1.10.2.2 18S PCR and PCR-RFLP

The small subunit (SSU) 18S rRNA gene is a multi-copy locus gene [~2251 base pairs (bp)] that is highly conserved, and frequently used as a marker in epidemiological studies (Stevens and Gibson, 1999). Numerous sequences of the gene can be obtained on databases such as GenBank. Despite these advantages, 18S PCR has been reported to be less sensitive. In a study by Gillingwater *et al.* (2010) in NKZN, a nested 18S PCR was reported to be less sensitive in detecting trypanosomes compared to a species-specific PCR targeting random amplified polymorphic DNA (RAPD) markers.

Polymerase chain reaction-restriction fragment length polymorphism assays have been developed to target the 18S ribosomal small subunit of trypanosomes (Geysen *et al.*, 2003; Delespaux *et al.*, 2003; Moti *et al.*, 2014). The 18S PCR-RFLP assays firstly amplify the *Trypanosoma* 18S rRNA gene fragment followed by treating the PCR product with restriction enzymes. The restriction enzymes cleave DNA at specific points and the fragments that are separated by electrophoresis according to sizes (Geysen *et al.*, 2003). Multiplex 18S PCR-RFLP has been used to detect *T. congolense*, *T. brucei*, *T. vivax* and *T. theileri* and mixed infections of these trypanosomes (Geysen *et al.*, 2003; Delespaux *et al.*, 2003; Osanyo and Majiwa, 2006; Odongo *et al.*, 2016). The PCR-RFLP assays are, however, time consuming.

1.10.2.3 Internal transcribed spacer PCR

Differentiation of trypanosome species has been made possible by the development of internal transcribed spacer (ITS) PCR which identifies the ITS1 region of the ribosomal ribonucleic acid (rRNA) (Picozzi *et al.*, 2008). This region is located between the 18S and 5.8S rRNA regions (Figure 4). The ITS1 region has trypanosome species length variation ranging between 300 bp to 800 bp and a high copy number, ranging between 100 and 200 copies per trypanosome genome (Desquesnes *et al.*, 2001; Njiru *et al.*, 2004a; Njiru *et al.*, 2004b). This

© University of Pretoria

is a useful marker as it allows trypanosome species to be differentiated from each other (Desquesnes *et al.*, 2001; Cox *et al.*, 2005).

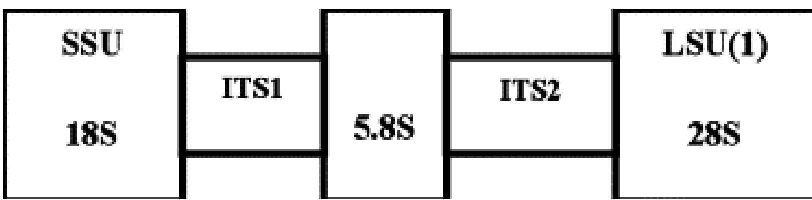


Figure 4: Ribosomal RNA gene structure (Cox *et al.*, 2005).

Nested ITS PCR assays have been developed for the detection of *Trypanosoma* species from field samples where the parasitaemia is low (Cox *et al.*, 2005). Previously used singleplex ITS PCR, which required primers to be designed for each specific species, has been replaced by an advantageous multiplex PCR that saves time and costs. Multiplex ITS PCR allows for the differentiation of *Trypanosoma* species simultaneously, in a single PCR, using only one set of primers (Table 1) that binds to a conserved region of the ITS1 gene (Njiru *et al.*, 2004a; Picozzi *et al.*, 2008). Primers targeting the ITS1 region of rRNA (Table 1), have been used to detect *Trypanosoma simiae*, *T. vivax*, *T. theileri*, *T. congolense* sub-species and *Trypanozoon* (Desquesnes *et al.*, 2001; Njiru *et al.*, 2004a; Njiru *et al.*, 2004b).

© University of Pretoria

Table 1: Oligonucleotide primers based on the ITS1 gene for detecting and distinguishing between *Trypanosoma* species (McLaughlin *et al.*, 1996; Njiru *et al.*, 2004a).

Primers	Trypanosome species	Amplicon size (bp)	Reference
ITS CF (5´-CCG GAA GTT CAC CGA TAT TG-3´) ITS BR (5´-TTG CTG CGT TCT TCA ACG AA-3´)	*T. congolense* Forest-type	710	(Njiru *et al.*, 2004a)
	T. congolense Savannah-type	700	
	T. congolense Kilifi-type	620	
	Trypanozoon: *T. brucei brucei*, *T. b. gambiense*, *T. b. rhodesiense* and *T. evansi*	480	
	T. simiae	400	
	Trypanosoma godfreyi	300	
	T. vivax	250	
Kin1(reverse) 5´-GCG TTC AAA GAT TGG GCA AT-3´ Kin2(forward) 5´-CGC CCG AAA GTT CAC C-3´	*T. congolense* Forest-type	780	(McLaughlin *et al.*, 1996)
	T. congolense Savannah-type	750	
	T. congolense Kilifi-type	680	
	Trypanozoon	540	
	T. theileri	455	
	T. simiae	435	
	T. vivax	305	

1.10.4 Real-time PCR

Quantitative real-time PCR (qPCR) assays offer several advantages over conventional PCR assays in that the analytical sensitivities of qPCR assays are much higher; quantification of parasite load is possible, and the assays can be multiplexed to allow for the simultaneous detection of multiple parasite species (Kaltenboeck and Wang, 2005).

Two qPCR assays were developed by Ahmed *et al.* (2015) to determine *T. congolense* Savannah-type and *T. b. brucei* populations in tsetse midgut. The qPCR assay for *T. b. brucei*

© University of Pretoria

targeted the single copy glycosyl-phosphatidylinositol-specific phospholipase C (GPI-PLC) gene and the qPCR assay for *T. congolense* targeted a mini-chromosome. These two qPCR assays detected both trypanosome species and quantified the level of infection within tsetse fly's midgut. The qPCR assays also indicated that *T. congolense* Savannah-type and *T. b. brucei* occurred as both single and mixed infections, with mixed infections indicative of competition between the two species.

Several quantitative real-time PCRs developed to detect other *Trypanosoma* species include; detection of *T. brucei* in human blood by targeting the 177 bp repeat satellite DNA (Becker *et al.*, 2004); detection of *T. cruzi* in tissues of infected mice targeting a kinetoplast minicircle DNA, in human tissue targeting the D7 region of the 24Sa rRNA gene, and in infected human blood targeting a 166 bp of the satellite DNA (Cummings and Tarleton, 2003, Freitas *et al.*, 2005; Piron *et al.*, 2007); and *T. evansi* in blood of infected rats targeting the ITS1 region of rRNA and in water buffalo blood targeting the Rode *Trypanozoon* antigen type (RoTat) 1.2 VSG gene, (Taylor *et al.*, 2008; Konnai *et al.*, 2009). Quantitative real-time PCR assays, targeting the ITS1 rRNA gene, were subsequently developed for the detection of *T. brucei*, *T. vivax* and *T. congolense* infections in cattle (Silbermayr *et al.*, 2013).

1.10.5 Cathepsin L

Cathepsin L (CATL) like genes are a multigene family that is arranged in tandem repeats copies and these genes encode for CATL-like cysteine proteases (Rodrigues *et al.*, 2010). Cathepsin L-like cysteine proteases are in the papain, C1 family (Rodrigues *et al.*, 2014). Phylogenetic analysis conducted by Cortez *et al.* (2009) on CATL-like genes of several trypanosomes revealed that trypanosomes had multiple CATL-like sequences which vary among species. The multiple copies and species-specific polymorphism have been useful in exploiting CATL-like sequences as potential genetic markers for sensitive and specific diagnostic tests, and the development of drugs and vaccines (Cortez *et al.*, 2009; Ortiz *et al.*, 2009; Rodrigues *et al.*, 2010).

An indirect ELISA for *T. vivax* based on the recombinant CATL antigen was able to distinguish between infected and non-infected animals (Eyssen *et al.*, 2018). Cathepsin L-like genes have also been successfully targeted by PCR for the specific and sensitive diagnosis of the following trypanosomes: *T. theileri* in buffaloes, cattle, cervids and tabanids samples (Rodrigues *et al.*, 2010, Pacheco *et al.*, 2018); *Trypanosoma trinaperronei* in white-tailed deer (Garcia *et al.*, 2020); *Trypanosoma rangeli* in human and wild animal samples (Ortiz *et al.*, 2009); and *T. vivax* in cattle samples (Cortez *et al.*, 2009). However, the low sensitivity of conventional PCRs which ranges between 0.001–0.02 parasites/μl (Desquesnes and Davila, 2002), creates a

© University of Pretoria

need for a more sensitive CATL diagnostic tool, and currently, there is no real-time PCR developed to target the CATL genes in trypanosomes. Hence, in this study, we describe the development of TaqMan MGB™ CATL qPCR assays for the specific and sensitive diagnosis of *T. brucei*, *T. theileri* and *T. congolense*.

1.11 Motivation

Over 50 million cattle and 173 million goats are at risk of *Trypanosoma* infections in sub-Saharan Africa and this economically hinders rural farmers who greatly rely on livestock. In NKZN, *Trypanosoma* infections are very common, and the disease has been reported to impact the productivity of cattle, milk and meat production, cattle growth and calving rates. Most of trypanosomosis research in South Africa has been on bovine and wildlife, and not much research has been done on small ruminants which sometimes serve as infection reservoirs. A better understanding of the prevalence of AAT in goats in NKZN will aid in improving control strategies for trypanosomosis in, cattle, small ruminant animals and the wildlife within the Hluhluwe-uMfolozi game reserve. It is also important to investigate the possible reservoir status of trypanosomes in goats to add to existing literature on AAT in small ruminants.

Although conventional PCR assays have been used for *Trypanosoma* diagnosis, they are time consuming, laborious and lack sensitivity. Real-time PCR assays are faster, they remove the need of end point measurement of PCR product and can quantify the DNA during amplification. The ITS qPCR assay has been shown to have a high sensitivity and specificity for *T. congolense*, *T. vivax* and *T. brucei*, however, cross-reactivity to other less characterized and highly prevalent non-pathogenic trypanosomes may compromise its use for molecular distinction and differentiation. Hence, there is a need to develop a qPCR assay based on an alternative gene for the distinction between *Trypanosoma* species.

The cathepsin L-like genes has been previously targeted in diagnostic, genotyping and phylogenetic studies of trypanosomes (Ortiz *et al.*, 2009; Rodrigues *et al.*, 2010; Eyssen *et al.*, 2018). Advancing the molecular diagnostic tools from conventional CATL PCRs to real-time PCRs, would improve detection of trypanosomes due to the high sensitivity and specificity of qPCRs in general and the high polymorphism of the CATL gene among trypanosomes. Therefore, three sensitive real-time PCR assays targeting the CATL-like gene of trypanosomes, were developed for the specific and sensitive detection of *T. congolense*, *T. brucei* and *T. theileri*.

© University of Pretoria

1.12 Aim and Objectives

Aim

The primary aim of the study was to survey AAT in cattle and goats in NKZN, and to develop CATL qPCR assays for the specific and sensitive diagnosis of *T. congolense*, *T. theileri* and *T. brucei*.

Study Objectives

i. To determine the *Trypanosoma* species in cattle and goats in NKZN using real-time PCR assays.
ii. To determine possible role that goats might have in maintaining and spreading *Trypanosoma* infections.
iii. To design primers and probes specific for the amplification of *T. congolense*, *T. brucei* and *T. theileri* infections in cattle blood samples.
iv. To compare performance of BCT, conventional ITS PCR, ITS qPCR assay and CATL qPCR assays in amplifying parasite DNA in cattle field samples.

© University of Pretoria

CHAPTER 2

MATERIALS AND METHODS

2.1 Study area

The present study was conducted on three diptanks in northern KwaZulu-Natal (NKZN) namely Ocilwane, Mvutshini and Ekuphindisweni. Mvutshini and Ekuphindisweni diptanks are under the Big 5 Hlabisa Municipality and Ocilwane diptank is under Mtubatuba Municipality (Table 2). Both municipalities fall within the Umkhanyakude District (Figure 5). Selection of the three diptanks was based on previous trypanosomosis prevalence data and the distribution of the diptanks in relation to their distance from the Hluhluwe-uMfolozi game reserve. The cattle that were sampled at these diptanks were selected based on the willingness of the owners to partake in the study.

2.2 Sampling

All cattle at each specific diptank were considered as one herd as they graze together and are handled using the same animal husbandry practice (Ntantiso *et al.*, 2014). Goats belonging to farmers or households that surround Mvutshini, Ocilwane, and Ekuphindisweni diptanks (Table 2) were sampled to determine possible *Trypanosoma* prevalence. A total of 384 goats and 60 cattle were sampled in this study from these tsetse infested areas, between March and April 2019.

Table 2: Sampling Sites in NKZN, (Ntantiso, 2012).

Site Identification	Site name	Coordinates
Diptank 1	Ekuphindisweni	26.57 S 32.46 E
Diptank 2	Mvutshini	28.07 S 32.09 E
Diptank 3	Ocilwane	28.26 S 32.00 E

© University of Pretoria

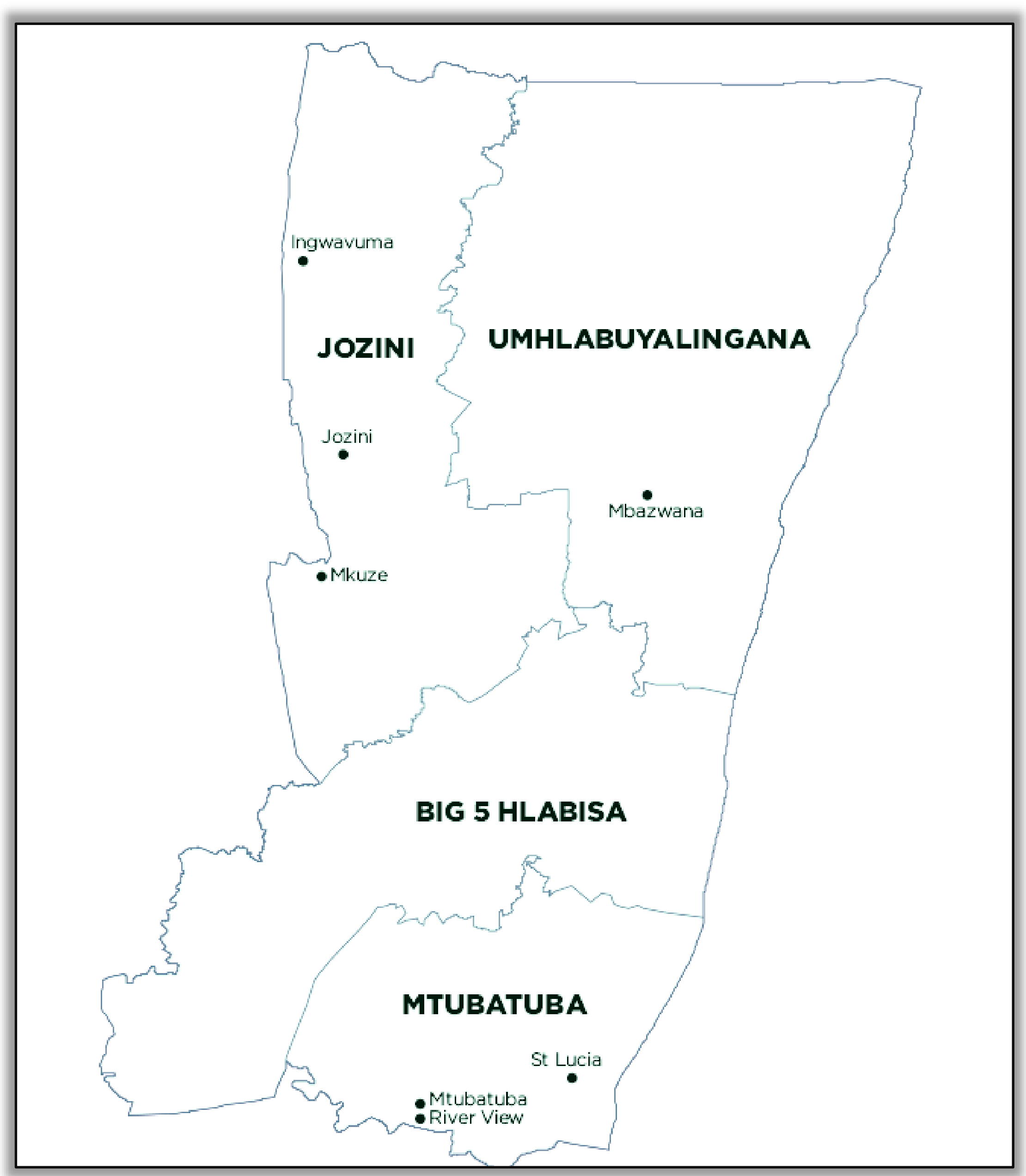


Figure 5: Map of the northern KwaZulu-Natal districts in South Africa.
1. Ekuphindisweni diptank 2. Mvutshini diptank 3. Ocilwane diptank.
Source: https://municipalities.co.za/map/1239/big-5-hlabisa-local-municipality

© University of Pretoria

2.2.1 Sample size determination

The study was conducted using 60 cattle from the three diptanks mentioned (Table 2). The sample size was determined as described in Thrusfield, (2007). Briefly, it was assumed that in an infinite population with a prevalence rate of 15%, a desired precision of 0.95 and confidence level of 95%, the estimated sample size at each diptank is 19. The sample size was increased to 20 cattle per diptank to provide an allowance for sample loss, bringing the total sample size to 60. Twenty (20) adult cattle per diptank were selected for sampling. The gender, body condition and treatment history of the cattle were recorded.

Goat samples were collected from communal farmers that bring their cattle to the selected diptanks. Samples (n=384) were only collected from goats belonging to owners willing to partake in the study. The sample size was estimated using formula $n = \frac{Z^2 P(1-P)}{d^2}$ (Thrusfield, 2007) with an assumed prevalence of 50%. Depending on the availability, between 8 and 15 animals were randomly sampled from each household/farmer. The gender, age, body condition and treatment history were recorded.

2.2.2 Sample collection and diagnostic test

Blood was collected from tail veins of adult cattle and the jugular veins of adult goats, into 10 mL vacutainer tubes containing EDTA (BD Vacutainer®; BD, Plymouth, UK). The blood samples were investigated for the presence of *Trypanosoma* parasites using the buffy coat technique (BCT). A small aliquot of the collected blood from each sample was transferred to microhaematocrit centrifuge capillary tubes that were sealed with Cristaseal (Marienfeld, Germany) and centrifuged in a haematocrit centrifuge. Centrifugation was done at 11 000 x g for 5 minutes to separate the plasma and blood cells. Packed cell volume (PCV) for all the samples was determined using a haematocrit tube reader, and cattle with PCV of 24% or less were considered anaemic (Van den Bossche, 2000).

After determining the PCV, the buffy coat layer was extruded onto a microscope slide and slides were examined for motile trypanosomes using a compound microscope at 40-times magnification. *Trypanosoma* species identification was based on the parasite motility and morphology.

The prevalence of *Trypanosoma* infection was calculated at each diptank as the percentage of the animals with trypanosome infections (herd average prevalence) (Van den Bossche and Rowland, 2001). The average anaemia for both cattle and goats at each diptank was calculated as the average of all anaemic animals (PCV ≤ 24%) at the particular diptank.

© University of Pretoria

(Ntantiso *et al.*, 2014). The herd average PCV (HA-PCV) was calculated for both cattle and goats as the average PCV at each sampling site (Van den Bossche and Rowland, 2001). The above values are important in the study as they are herd health status indicators (Trail *et al.*,1991; Ntantiso *et al.*, 2014).

2.3 Molecular analysis

2.3.1 Extraction of DNA from blood samples

The DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, 20 µl of the Qiagen Protease was added to a 1.5 mL microcentrifuge tube followed by addition of 200 µl of the blood sample. The lysis buffer, Buffer AL (200 µl) was added to the mixture, which was then pulse-vortexed and incubated at 56 °C for 10 minutes. This was followed by the addition of 200 µl of absolute alcohol and mixing by pulse-vortexing. The mixture was transferred to the Qiagen Mini spin column and centrifuged at 6000 x g for 1 minute. The filtrate was discarded, and the wash buffer, Buffer AW1 was added to the Qiagen Mini spin column and centrifuged at 6000 x g for 1 minute. This was followed by a second wash using Buffer AW2 and centrifugation at 20 000 x g for 3 minutes. The DNA was eluted in 100 µl of elution buffer, Buffer AE and stored at -20 °C until further required.

2.3.2 Molecular identification of trypanosomes using ITS PCR

All samples (Goat: n = 384 and Cattle: n = 60) were tested for the presence of *Trypanosoma* parasite DNA using the conventional polymerase chain reaction (PCR) targeting the internal transcribed spacer-1 (ITS) region of the rRNA gene. Briefly, published ITS1 CF forward (5´CCGGAAGTTCACCGATATTG3´) and ITS1 BR reverse (5´TGCTGC GTTCTTCAACGAA3´) primers were used to produce amplicons of sizes between 250 base pairs (bp) – 710 bp, depending on the trypanosome species (Njiru *et al.*, 2004a). The PCR was carried out in a reaction volume of 25 µl containing 12.5 µl One Taq® Quick -Load® 2X Master Mix with Standard Buffer (New England, Biolabs® Inc, USA), 10 µM of ITS1 CF primer, 10 µM of ITS1 BR primer, 9 µl of nuclease-free water and 2.5 µl extracted DNA. Reactions were performed using GeneAmp® PCR System 9700 (Applied Biosystems, South Africa). The thermocycling conditions included an initial denaturation of 3 minutes at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C, with a final extension of 10 minutes at 72 °C. DNA from *Trypanosoma congolense*, *Trypanosoma brucei* and *Trypanosoma vivax*, obtained from the University of Pretoria (UP),

© University of Pretoria

Department of Veterinary Tropical Diseases (DVTD), were used as positive controls and the negative control was a no template control.

2.3.3 Agarose Gel Electrophoresis

The amplicons were visualised by 2% agarose gel electrophoresis. The 2% agarose gel was prepared by heating a mixture of 2 grams agarose (Bioline, Canada) and 100 ml 1x Tris-acetate EDTA (TAE) electrophoresis buffer (40 pmol/µL Tris-acetate, 1 pmol/µL EDTA) and stained using 5 µl of ethidium bromide (Bio-rad, USA). The PCR products (5 µl) were loaded into wells and separated by electrophoresis at 120V for 30 minutes. A 100 bp DNA ladder was added to every gel for fragment size determination. The gel was visualized under UV light.

2.3.4 ITS real-time PCR assay

Extracted DNA from cattle blood samples were screened for the presence of *Trypanosoma* DNA using published genus-specific PCR primers targeting the ITS1 gene (Silbermayr *et al.*, 2013). One set of universal primers and three probes, each specific for *T. congolense*, *T. vivax* and *T. brucei* were designed to amplify a 120 bp region within the ITS1 gene (Table 3). The assay was modified for use on the LightCylcer® 480 PCR platform at the ARC-OVR Epidemiology, Parasites and Vectors Department. For the simultaneous detection of all three parasites in a multiplex assay, each of the species-specific probes were labelled with different fluorescent dyes (Table 3).

Briefly, quantitative real-time PCR assays (qPCR) were performed using the LightCycler® 480 PCR System (Roche Molecular Diagnostics, Germany). Reactions were performed in a 20 µl volume containing 10 µl KAPA Probe Fast Universal Master Mix (Kapa Biosystems, South Africa), 0.9 µM Tryps_KS-forward primer, 0.9 µM Tryps_KS-reverse primer, 0.25 µM of each of the species-specific probes, 2.5 µl of target DNA and 3.5 µl of nuclease-free water. Cycling conditions were as follows: enzyme activation at 95 °C for 30 seconds followed by 45 cycles of 5 seconds at 95 °C and 40 seconds at 68 °C. Data were analysed with the LightCycler® 480 software using the Second Derivative Maximum Method.

© University of Pretoria

Table 3: Universal primers and genus-specific ITS1 real-time probes for detecting African animal trypanosomosis (AAT) (Silbermayr *et al.*, 2013).

Name	Primer/Probe	Product size
Tryps_KS-for	5´- CGT GTC GCG ATG GAT GAC TT -3´	120 bp
Tryps_KS-rev	5´- CAA ACG GCG CAT GGG AG -3´	120 bp
Tryps_KS-T.cong-p	CY5 5´- TTG CAG AAT CAT CAC ATT GCC CAA TCT TTG -3´ BHQ1	
Tryps_KS-T.brucei-p	FAM 5´- TGC GAT AAG TGG TAT CAA TTG CAG AAT CAT TTC A -3´ BHQ1	
Tryps_KS-T.vivax-p	HEX 5´- ATG ACC TGC AGA ACC ACT CGA TTA CCC AGT -3´ BHQ1	

2.4 Development of cathepsin L-like (CATL) real-time PCR assay

2.4.1 Primer and Probes design

A multiple sequence alignment of 36 *Trypanosoma spp* cathepsin L (CATL) reference sequences retrieved from GenBank (*T. brucei* AC: EU753820, EU753821, EU753822; *Trypanosoma theileri* AC: GU299366, HQ543075, HQ543074, GU299414, GU299407; *T. congolense* AC: KF413956, KF413961, KF414041, KF414001, KF414025, KF413898, KF413923, KF413934, KF413941, KF413987, KF413977, KF413949, KF414048, KF414051, KF414043, KF413965, KF414042, KF413995, KF413984, KF413964, KF413960, KF413952, KF413957; *T. vivax* AC: EU753814; *Trypanosoma simiae* AC: KF414037; *Trypanosoma cruzi* AC: M84342; *Trypanosoma rangeli* AC: L38512, L38514) (https://www.ncbi.nlm.nih.gov/), was created using the MUSCLE algorithm (Edgar, 2004). A TaqMan minor groove binder (MGB™) real-time PCR assay was designed for *T. congolense*, *T. brucei* and *T. theileri* using the Primer express software v3.0 (Applied Biosystems) and evaluated using BLAST (Altschul *et al.*, 1990). Three species-specific forward primers; one genus-specific reverse primer and three TaqMan MGB™ probes were designed (Table 4) to amplify fragments of the CATL-like gene from *T. congolense* (116 bp), *T. brucei* (98 bp) and *T. theileri* (137 bp). Primers were synthesized by Inqaba

© University of Pretoria

Biotechnologies (South Africa) and TaqMan MGB™ probes were synthesized by Integrated DNA Technologies, Inc. (IDT), (USA). Quantitative PCR assays were performed in MicroAmp™ optical 96-well plates using the StepOnePlus™ real-time instrument (Applied Biosystems™, USA).

Reactions were prepared in a final volume of 20 µl containing 10 µl KAPA Probe Fast Universal Master Mix (Kapa Biosystems, South Africa), 0.9 µM of each CATL species-specific forward primer and genus-specific reverse primer, 0.25 µM TaqMan MGB™ species-specific probe, and 2.5 µl target DNA. The qPCR cycling conditions were as follows: activation at 95 ºC for 20 seconds followed by 40 cycles of 1 second at 95 ºC and 30 seconds at 60 ºC. Results were analysed using the StepOnePlus™ software (v2.3).

Table 4: Primers and probes designed to amplify *Trypanosoma* CATL-like gene.

Name	**Primer/Probe**	**Size**	**Product size**
TcongCATL_fwd	5´- CTA CAC GGG CGG AGT GTT G -3´	19	116 bp
TtheilCATL_fwd	5´- CGA CGC CAA CAG CTT CCT -3´	18	137 bp
TbrucCATL_fwd	5´- GACTTCATGCACCTCCGAGC -3´	20	98 bp
TrypsCATL_reverse	5´- CCC GAG AGT TCT TGA TGA TCC A -3´	22	
TcongCATL_probe	FAM 5´- TGG GGT ATG ACG ACA CAA - 3´ MGB	18	
TtheilCATL_probe	VIC 5´- GGC TAC GAC GAC AGC A - 3´ MGB	16	
TbrucCATL_probe	NED 5´- GAT AAT AGC AAT CCA CCC - 3´ MGB	18	

2.4.2 Analytical sensitivity of the CATL qPCR assays.

Plasmid standards were generated for each parasite species to determine sensitivity of each CATL qPCR assay.

© University of Pretoria

2.4.2.1 Amplification of the CATL-like gene

The CATL gene was amplified from each species-specific positive control DNA of *T. theileri*, *T. brucei*, and *T. congolense* obtained from UP DVTD, molecular diagnostics laboratory using the species-specific forward and genus-specific reverse primers described previously (Table 4).

Reactions were set up in a final reaction volume of 25 µl containing 12.5 µl One Taq® Quick -Load® 2X Master Mix (New England, Biolabs® Inc, USA), 10 µM of CATL species-specific forward primer, 10 µM of CATL genus-specific reverse primer, 9 µl of nuclease-free water and 2.5 µl of the DNA control. GeneAmp® PCR System 9700 (Applied Biosystems, South Africa) was used for PCR cycling conditions included: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds, with a final extension step at 72°C for 10 minutes.

2.4.2.2 PCR product purification

The PCR products purification was carried out the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, binding buffer containing isopropanol was added to the PCR product. The sample was loaded into a spin column in a wash tube and centrifuged at 10 000 × g for 1 minute. A wash buffer was added to the spin column and the sample was centrifuged at 10 000 × g for 1 minute. Residual ethanol was removed by performing an additional centrifugation step at maximum speed for 3 minutes. The purified PCR product was eluted in 50 µl Elution buffer and stored at 4 °C.

2.4.2.3 Cloning of the *Trypanosoma* CATL-gene

The CATL PCR fragments amplified from *T. congolense*, *T. theileri* and *T. brucei* were cloned into the pJET1.2/blunt cloning vectors (Figure 6) using a CloneJET PCR Cloning Kit (Thermo Scientific, USA), according to the manufacturer's instructions. Ligation was performed in a 3:1 insert-to-vector ratio. A 10 µl ligation reaction was prepared containing 50 ng pJET1.2/blunt Cloning Vector (Figure 6), 2X reaction buffer, 3 volumes of the purified PCR product and 1 volume of T4 DNA ligase. The ligation reaction mixture was gently mixed and incubated for 5 minutes on ice.

The ligated plasmids were transformed into competent *Escherichia coli* JM109 cells (Zymo Research, USA). A volume of 50 µl of *E. coli* competent cells was added to 2 µl of each

© University of Pretoria

ligation reaction and the mixture was incubated on ice for 30 minutes. This was followed by heat-shock treatment at 42 °C for 45 seconds and immediate incubation on ice for 2 minutes. A volume of 250µl Super Optimal broth with Catabolite repression (SOC) medium was added to each reaction and incubated at 37 °C for 1 hour in a shaking incubator with a speed of 250 rpm. Transformants were screened on Luria Bertani (LB) plates containing 100 µg/mL ampicillin. The plates were incubated overnight at 37 °C. Recombinant colonies were selected and individually inoculated into 5 ml LB medium supplemented with 50 µg/mL ampicillin. Following an overnight incubation at 37 °C the bacterial cells were subjected to plasmid DNA isolation.

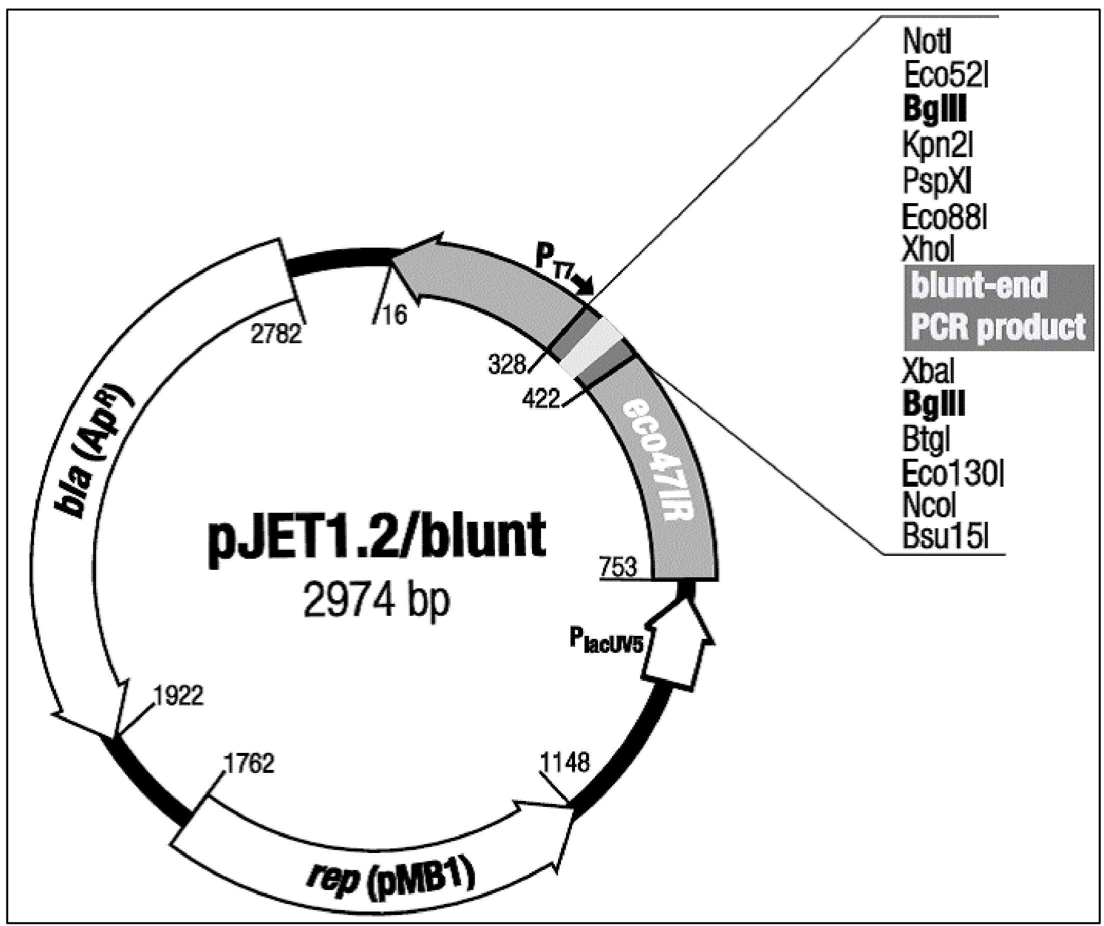


Figure 6: The pJET1.2/blunt vector map.
Source:http://reports.ias.ac.in/report/13592/cloning-3-untranslated-region-of-dengue-virus-in-pjet12blunt-cloning-vector

Plasmids were isolated using the High Pure Plasmid Isolation Kit (Roche, Switzerland) as per the manufacturer's protocol. The bacterial culture was centrifuged at 6000 x g for 30 seconds. The resulting pellet was resuspended in 250 µl of suspension buffer containing

© University of Pretoria

RNase. The cells were then lysed by the addition of 250 µl lysis buffer. The mixture was incubated for 5 minutes at room temperature before adding 350 µl of cold binding buffer. After mixing, by gently inverting the tube, the sample was incubated for 5 minutes on ice and then centrifuged for 10 minutes at maximum speed. The clear supernatant was transferred into a High Pure filter tube placed into a collection tube and centrifuged at maximum speed for 1 minute. The flow-through was discarded; 500 µl of wash buffer I was added to the column and the sample was centrifuged at 13 000 x g for 1 minute. This was

followed by a second wash using 700 µl of wash buffer II. After discarding flow-through the tube was centrifuged for an additional minute at 13 000 x g in order to remove all residual ethanol. The filter tube was placed into a clean Eppendorf tube and 50 µl of Elution Buffer was added. The tube was centrifuged at 13 000 x g for 1 minute. Isolated plasmid DNA controls were stored at 4 °C. Plasmid DNA was sent to Inqaba Biotechnologies (South Africa) for capillary DNA sequencing. The plasmid inserts were sequenced using pJET1.2 forward sequencing primer (5`- CGACTCACTATAGGGAGAGCGGC3`) and pJET1.2 reverse sequencing primer (5`- AAGAACATCGATTTTCCATGGCAG3`). Sequences were assembled and edited using the CLC Genomics Workbench (CLC Bio version 8.0.1). Multiple sequence alignments were performed using the MUSCLE algorithm (Edgar, 2004) and sequences were queried against previously published CATL gene sequences using BLAST (Altschul *et al.*, 1990).

2.4.2.4 Preparing the standard dilution series

A single recombinant clone for each parasite (*T. theileri*, *T. congolense* and *T. brucei*) was selected and the DNA concentration determined using the Xpose DNA/RNA Analysis (Trinean, Belgium). The copy number of each parasite-specific CATL-plasmid construct was calculated using the following formula:

$$\text{number of copies (molecules)} = \frac{\text{X (ng)} * 6.0221 \text{ x } 10^{23}}{\text{N (bp)} * 1 \text{ x } 10^{9} \text{ x } 660}$$

Where:

X = amount of amplicon (ng)

N = length of dsDNA amplicon (bp)

660 = average mass of 1 bp dsDNA (g/mole)

$6.0221 \text{ x } 10^{23}$ = Avogadro's constant

$1 \text{ x } 10^{9}$ = conversion factor

© University of Pretoria

The concentration of each plasmid construct was adjusted to 10^9 copies/µl. A ten-fold serial dilution (10^9-10^0 copies/ul) was prepared and qPCR amplifications (stated in section 2.4) of each standard dilution series was repeated in triplicate. Linear regression curves were generated by plotting the mean cycle threshold (Ct) values against the mean log copy number. The amplification efficiency (E) of each assay could be determined from the curve using the equation $E=10^{1/-s} - 1$. Acceptable E values were between 90%-110%.

2.4.2.5 CATL qPCR Amplification conditions

Amplification was done in a final volume of 20 µl containing 10 µl KAPA Probe Fast Universal Master Mix (Kapa Biosystems, South Africa), 0.9 µM of each CATL species-specific forward primer and genus-specific reverse primer, 0.25 µM TaqMan MGB™ species-specific probe, 2.5 µl DNA and 3.65 µl of nuclease-free water. The qPCR cycling conditions were as follows: activation at 95 ºC for 20 seconds followed by 40 cycles of 1 second at 95 ºC and 30 seconds at 60 ºC. Cycling was performed in a StepOnePlus™ (Applied Biosystems™, USA) and the results were analysed using the StepOnePlus™ software (v2.3).

2.4.3 Analytical specificity of the CATL qPCR assays

The intraspecies analytical specificity of each assay was determined by testing DNA samples from other *Trypanosoma* species expected to occur in livestock. For *T. congolense* CATL qPCR assay, the analytical specificity was determined by using positive control DNA from *T. brucei*, *T. theileri* and *T. vivax*; for *T. brucei* CATL qPCR assay analytical specificity *T. congolense*, *T. theileri* and *T. vivax* positive control DNA were used; and for *T. theileri* CATL qPCR assay analytical specificity *T. brucei, T. vivax* and *T. congolense* positive control DNA were used. The interspecies analytical sensitivity of the CATL qPCR assays was also determined by testing DNA samples from other parasites known to infect cattle, such as *Babesia bovis*, *Babesia bigemina* and *Theileria parva* obtained from UP DVTD molecular diagnostics laboratory.

2.4.4 Evaluation of cattle and goats' samples using the CATL qPCR assays

Field samples collected from cattle (n =60) and 10% of the goats (n=39) were used to evaluate the diagnostic ability of each of the developed *Trypanosoma* CATL qPCR assays in detecting parasite DNA.

© University of Pretoria

2.5 Statistical analyses

Microsoft® Excel spread sheet was used for data entry and the data was transferred to the Statistical Package for Social Sciences (SPSS) version 26.0 software for statistical analysis. One-way analysis of variance (ANOVA) was used to analyse differences in the HA-PCV of goats and cattle between the three diptanks. Tukey post hoc test was used to test for the difference in means among diptanks. The independent t-test was used to test association between: a) the average PCV of infected and non-infected animals b) the average PCV of anaemic and non-anaemic animals. The Fisher's exact test was performed to determine differences between BCT, ITS PCR, ITS qPCR and CATL qPCR. Confidence intervals of 95% and p-value of 0.05 were used. *Trypanosoma* infection rate was calculated as follows:

$$\text{infection rate} = \left(\frac{\text{number of cattle infected}}{\text{number of cattle sampled}}\right) \times 100\ .$$

© University of Pretoria

CHAPTER 3

RESULTS

3.1 Parasitological detection of trypanosomes

One-way analysis of variance (ANOVA) showed a statistically significant difference between the herd average packed cell volume (HA-PCV) of the three diptanks ($F(2,57)=4.57$; $p=0.017$). Tukey post hoc test revealed that there was no significant difference between HA-PCV of cattle at Ocilwane (28.9±4.50%, $p=1.00$) and Ekuphindisweni (28.9±4.50%, $p=1.00$). However, the HA-PCV of Mvutshini (32.4±3.98%, $p=0.035$) was significantly higher than that observed at both Ocilwane and Ekuphindisweni (Table 5).

The difference in HAA among the three diptanks was not statistically significant, $p>0.05$. Nine of the 60 cattle (15%) were anaemic. Four anaemic cattle [herd average anaemia (HAA)=20%] were detected at Ekuphindisweni (n=20), 3 anaemic cattle (HAA=15%) and 2 anaemic cattle (HAA=10%) were detected at Ocilwane (n=20) and Mvutshini (n=20), respectively (Table 6). The average PCV in anaemic cattle was significantly lower (22.7±2.80%) than in the non-anaemic cattle (31.4±3.40%) ($t(58)=-7.182$, $p<0.0005$).

Using the buffy coat technique (BCT), 7 (11.7%) of the 60 cattle were positive for trypanosomes and only *Trypanosoma congolense* was detected. The highest number of infections were recorded at Mvutshini and Ekuphindisweni, where 3 (15%) *Trypanosoma* positive animals were detected at both diptanks. Although only one (5%) *Trypanosoma* positive animal could be detected at Ocilwane (Table 6), there was no statistically significant difference between the infection rate of *Trypanosoma* at all three diptanks. There was also no statistically significant difference between the average PCV of the *Trypanosoma* infected cattle as detected by BCT (27.1±7.10%) and the average PCV of the uninfected cattle (30.5±4.10%) ($t(58)=1.837$, $p=0.071$). Four (57.1%) of the BCT positive cattle were anaemic and 9.4% (5 of 53) of the uninfected cattle were anaemic (Table 6).

None of the goats' samples tested positive for of trypanosomes by BCT. However, the one-way ANOVA showed a significant difference of HA-PCV ($F(2, 381)= 4.057$; $p<0.05$) between the three diptanks. Goats screened at Mvutshini (n=128) had a statistically significant higher HA-PCV (28.9±5.80%, $p=0.025$), than goats screened at Ekuphindisweni (n=128). There was no statistically significant difference of HA-PCV between Ocilwane (28.7±4.90%) and

© University of Pretoria

Mvutshini ($p=0.935$), as well as, no statistically significant difference between Ekuphindisweni HA-PCV (27.3±3.70%) and Ocilwane HA-PCV ($p=0.061$). Eighty-nine (23.2%) of the 384 goats sampled were anaemic. There were 37 anaemic goats (HAA=28.9%) at Mvutshini, 31 (HAA=24.2%) at Ekuphindisweni and 21 (HAA=16.4%) at Ocilwane (Figure 7). The independent t-test showed that the average PCV was significantly lower in the anaemic group (22.16±2.50%) than in the non-anaemic group (30.17±3.80%) ($t(382)=-18.867$, $p<0.0005$).

Table 5: Trypanosome infection rates, HA-PCV and HAA in cattle at the three diptanks.

Site name	Number of cattle	Infection rate (%)	HA-PCV (%±S.D)	HAA (%)
Ocilwane	20	5	28.9±4.50 [a,b]	15
Mvutshini	20	15	32.4±3.98 [b]	10
Ekuphindisweni	20	15	28.9±4.50 [a,b]	20
Significance		p>0.05	p<0.05	p>0.05

[a]The significant difference between Ocilwane and Ekuphindisweni HA-PCV, p=1.00.
[b]The significant difference between Ocilwane/Ekuphindisweni and Mvutshini HA-PCV, p=0.035.

© University of Pretoria

Table 6: Average PCV and anaemia observed at the three diptanks in infected and non-infected groups of cattle.

Site name	Number of cattle	Number of infected	PCV (%±S.D)		HAA (%)	
			Infected	Uninf-ected	Infected	Uninf-ected
Mvutshini	20	3	26.7±4.60	33.4±2.98	66.7	0
Ekuphindi-sweni	20	3	31.3±6.40	28.5±4.20	33.3	17.6
Ocilwane	20	1	16	29.6±3.40	100	10.5
Total	60	7	27.1±7.10	30.5±4.1	57.1	9.4

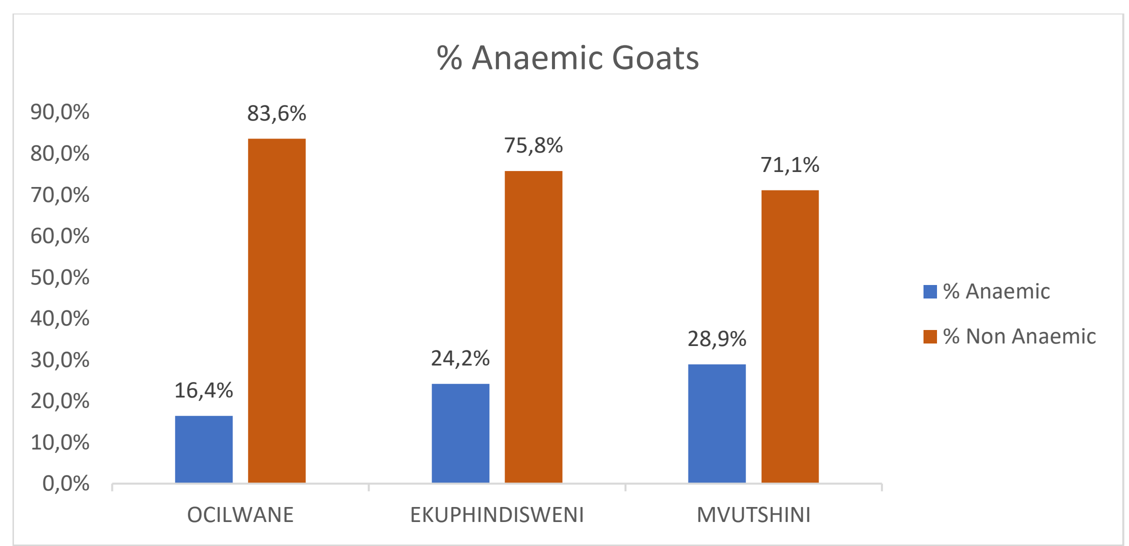


Figure 7: Bar chart showing the percentage of anaemic and non-anaemic goats' samples at each diptank (n=128).

© University of Pretoria

3.2 Molecular analysis

3.2.1 Molecular identification of trypanosomes using conventional ITS PCR

The ITS PCR assay was used to detect *Trypanosoma* parasites in cattle and goat samples. The expected amplicons are between 250 base pairs (bp) -710 bp. All the DNA samples that produced 620 bp-710 bp amplicon sizes on agarose gel were considered *T. congolense* positive, while those that produced amplicon sizes of ~480 bp and ~250 bp were considered positive for *T. brucei* and *T. vivax*, respectively. The DNA samples that produced no amplicons visible on agarose gel were considered to be trypanosome negative. While none of the goat samples tested positive for *Trypanosoma* parasites, 5 (8.3%) of the cattle samples (samples: 17, 32, 39, 40 and 47) produced amplicons between 620 bp - 710 bp, which indicated *T. congolense* infections (Figure 8). The ITS PCR assay did not detect any *T. brucei* or *T. vivax* infections in the cattle.

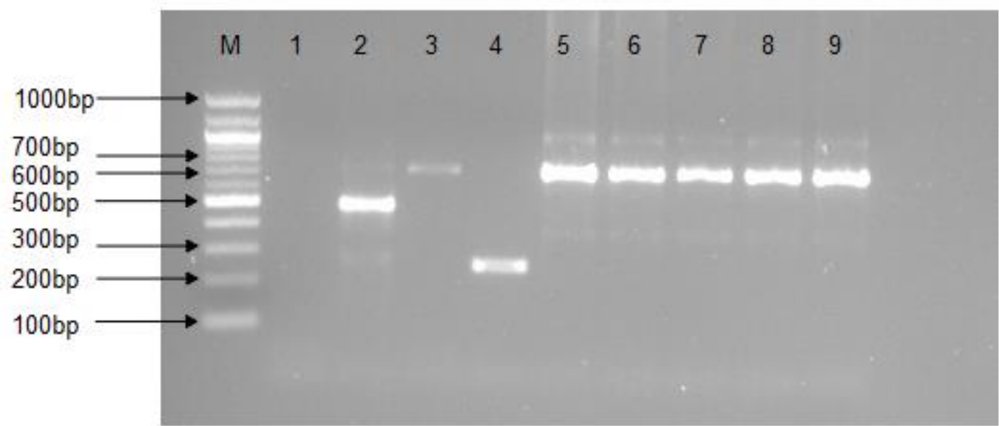


Figure 8: Agarose gel showing bands obtained from ITS PCR amplification using primers ITS1 CF and ITS1 BR.
Lane M represents a 100bp marker. Lanes: 1=Negative, 2=*T. brucei* positive control, 3=*T. congolense* positive control, 4=*T. vivax* positive control. Lanes 5, 6, 7, 8 & 9 represents cattle blood samples 17, 32, 39, 40 & 47, respectively, which all tested positive for *T. congolense*.

© University of Pretoria

3.2.2 Detection of trypanosomes using the ITS real-time PCR assay

According to Silbermayr *et al.* (2013) the cut off quantification cycle (Ct) value of this assay was 38 and any DNA samples with Ct value of >38 was regarded as negative. Eighteen of the 60 cattle (30%) were positive for trypanosome parasites. Only *T. congolense* and *T. brucei* were detected by ITS qPCR and no mixed infections were observed in the cattle. Six of the 60 cattle (10%) were positive for *T. congolense* and 20% (12/60) of the cattle were positive for *T. brucei*.

3.3 Development of the CATL qPCR assays

3.3.1 *In silico* analysis of the designed CATL qPCR primers and probes

The specificity of the primers and probes designed for each *Trypanosoma* species, were each tested *in silico* using BLAST analysis. Alignment of the genus-specific *Trypanosoma* CATL reverse primer (TrypsCATL_reverse) with other *Trypanosoma* CATL sequences, showed that the primer was conserved from position 463 of the *Trypanosoma* CATL sequences from GenBank (Figure 9, 10, 11). Seven extra nucleotide bases, based on the *Trypanosoma* sequences, were added to the reverse primer sequence to increase specificity. BLAST analysis further confirmed that the designed primer showed 100% sequence identity to CATL-like sequences from *T. congolense*, *T. brucei* and *T. theileri*.

The forward primer and probe sequences were species-specific and were developed for each *Trypanosoma* species. A Clustal W alignment of *T. congolense* CATL reference sequences retrieved from GenBank showed that the *T. congolense* forward primer (TcongCATL_fwd) binds at positions 369-387 and the *T. congolense* probe (TcongCATL_probe) binds at positions 431-448 (Figure 9). BLAST analysis showed the TcongCATL_fwd primer and TcongCATL_probe had 100% sequence coverage and identity to *T. congolense* CATL protein gene sequences available on GenBank.

Alignment of the *T. theileri* forward primer and probe sequences (TtheilCATL_fwd and TtheilCATL_probe) to the *T. theileri* CATL sequences available on GenBank showed that the sequences were conserved from positions 348-365 and 433-448, respectively (Figure 10). The TtheilCATL_probe and TtheilCATL_fwd showed sequence coverage and identity to *T. theileri* CATL sequences available on GenBank. Similarly, the *T. brucei* forward primer and probe sequences (TbrucCATL_fwd and TbrucCATL_probe) were 100% conserved at positions 387-406 and 442-459 (Figure 11), and showed 100% sequence identity to CATL sequences of *T. brucei* available on GenBank.

© University of Pretoria

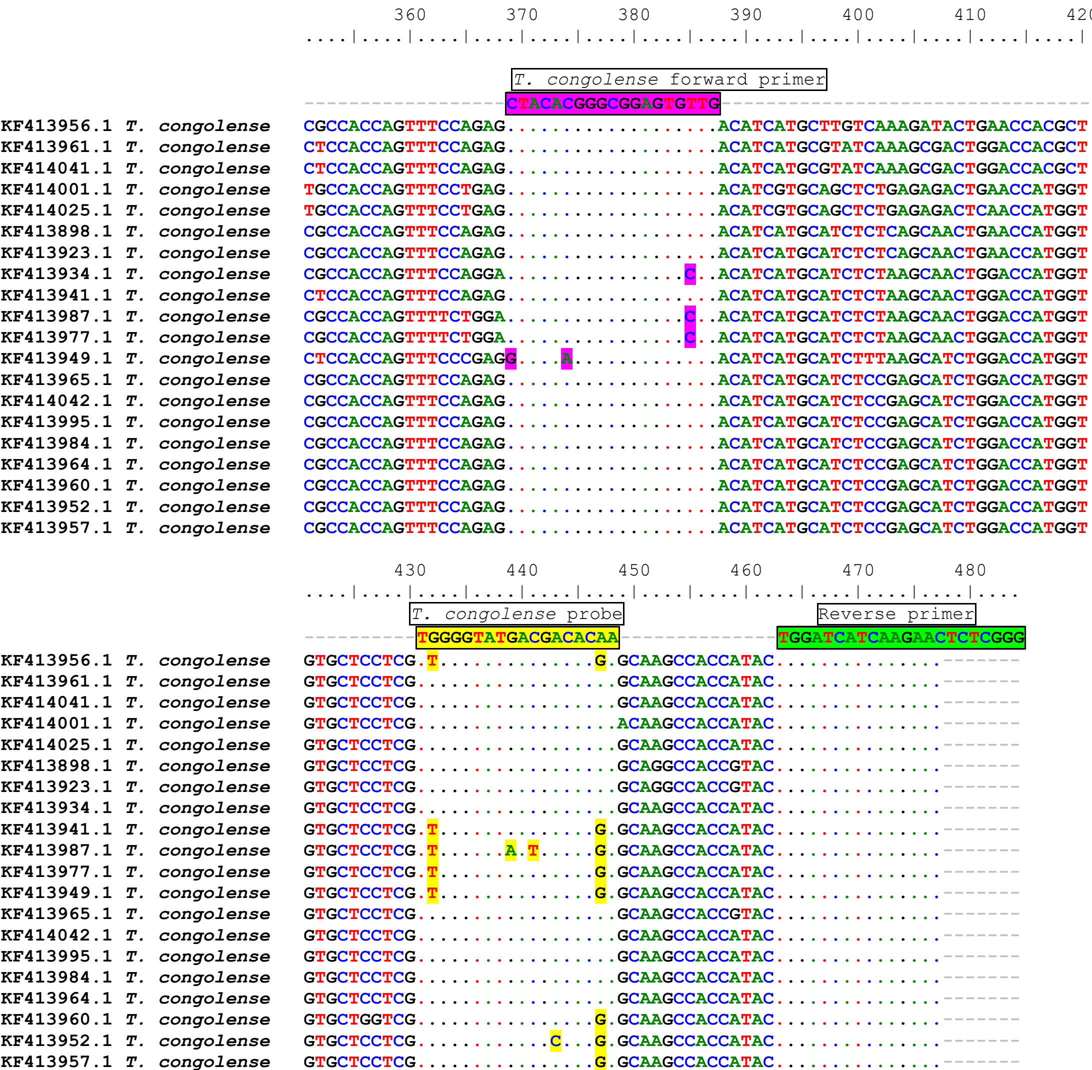


Figure 9: Nucleotide sequence alignment of the target region of the *T. congolense* CATL-specific qPCR assay in the CATL gene of published *T. congolense* sequences. The sequence of the species-specific forward primer is highlighted in pink, while the genus-specific reverse primer is highlighted in green. The species-specific *T. congolense* probe sequence is indicated in yellow and sequence differences between sequences in the forward primer and probe regions are also highlighted in the same colours used to indicate the oligonucleotides.

© University of Pretoria

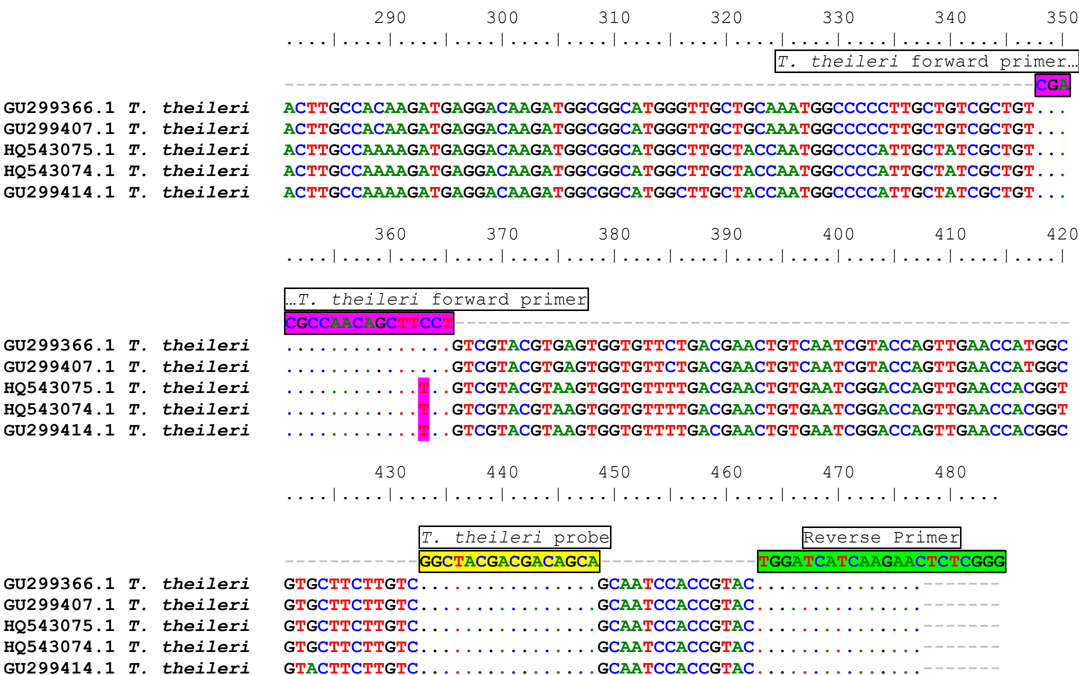


Figure 10: Nucleotide sequence alignment of the target region of the *T. theileri* CATL-specific qPCR assay in the CATL gene of published *T. theileri* sequences. The sequence of the species-specific forward primer is highlighted in pink and sequence differences between sequences in the forward primer region is also highlighted in pink. The species-specific *T. theileri* probe sequence is indicated in yellow and the genus-specific reverse primer is highlighted in green.

© University of Pretoria

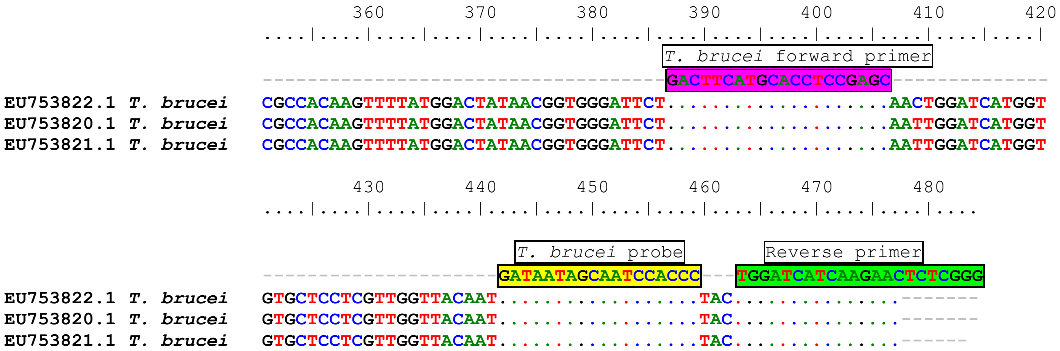


Figure 11: Nucleotide sequence alignment of the target region of the *T. brucei* CATL-specific qPCR assay in the CATL gene of published *T. brucei* sequences. The sequence of the species-specific forward primer is highlighted in pink and sequence differences between sequences in the forward primer region is also highlighted in pink. The species-specific *T. theileri* probe sequence is indicated in yellow and the genus-specific reverse primer is highlighted in green.

3.3.2 Efficiency, specificity and sensitivity of the developed species-specific *Trypanosoma* CATL qPCR assays

The developed CATL qPCR assays were able to detect and differentiate between the three *Trypanosoma* species investigated in this study. Primers TbrucCATL_fwd , TtheilCATL_fwd, TcongCATL_fwd and TrypsCATL_reverse were used to amplify regions of the CATL gene from *T. brucei* (98 bp), *T. theileri* (137 bp) and *T. congolense* (116 bp) DNA controls prior to cloning into the pJET1.2/blunt cloning vectors (Figure 12).

© University of Pretoria

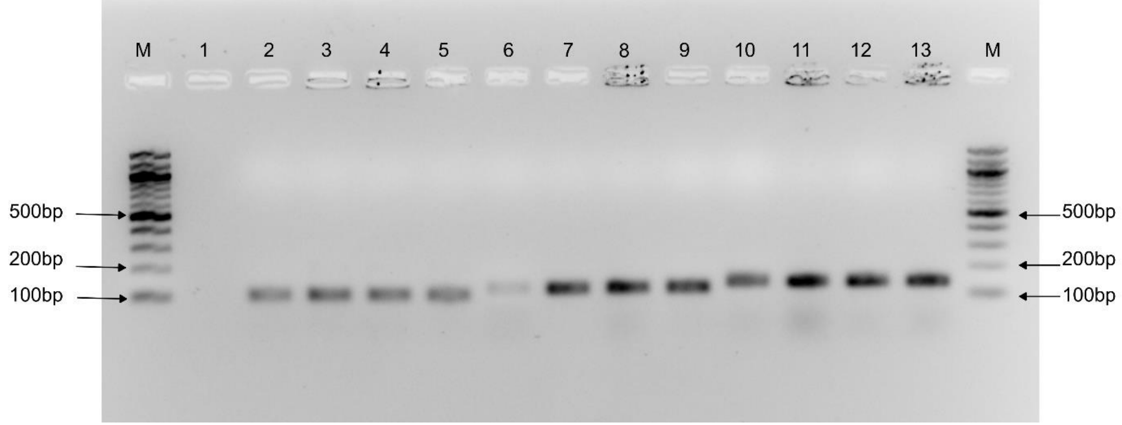


Figure 12: PCR analysis of the recombinant clones using the *Trypanosoma* species-specific CATL-forward and genus-specific CATL-reverse primers designed for the qPCR assays.
Lanes: M= Quick-Load® 100 bp DNA Ladder (New England Biolabs); 1= negative control, lanes 2-5= *T. brucei* CATL plasmid DNA for clone Tb_PC1, Tb_PC2, Tb_PC3 and Tb_PC4, respectively; lanes 6-9= *T. congolense* CATL plasmid DNA for clone Tc-_PC1, Tc_PC2, Tc_PC3 and Tc_PC4; lanes 10-13= *T. theileri* CATL plasmid DNA for clone Tt_PC1, Tt_PC2, Tt_PC3 and Tt_PC4.

3.3.3 Sensitivity and efficiency of the CATL qPCR assays

Regression analysis of each of the developed CATL qPCR assays indicated a linear correlation of log copy number against Ct values. The amplification efficiency in detecting parasite DNA was 99.2%, 97.2% and 94.3%, with a correlation coefficient (R^2) value of 0.988, 0.996 and 0.991 for *T. congolense*, *T. brucei* and *T. theileri*, respectively (Figure 13). The *T. congolense* CATL qPCR assay could detect template DNA in the range of 10^9 copies/reaction to 10^2 copies/reaction, which corresponded to Ct values from 11.3 to 35.9. The amplification range for *T. brucei* extended from 10^9 copies/reaction to 10^1 copies/reaction with average Ct values ranging from 12.8 to 38.45. The detection limit of the *T. theileri* CATL qPCR assay was 10^2 copies/reaction which corresponded with Ct value of 37.97.

© University of Pretoria

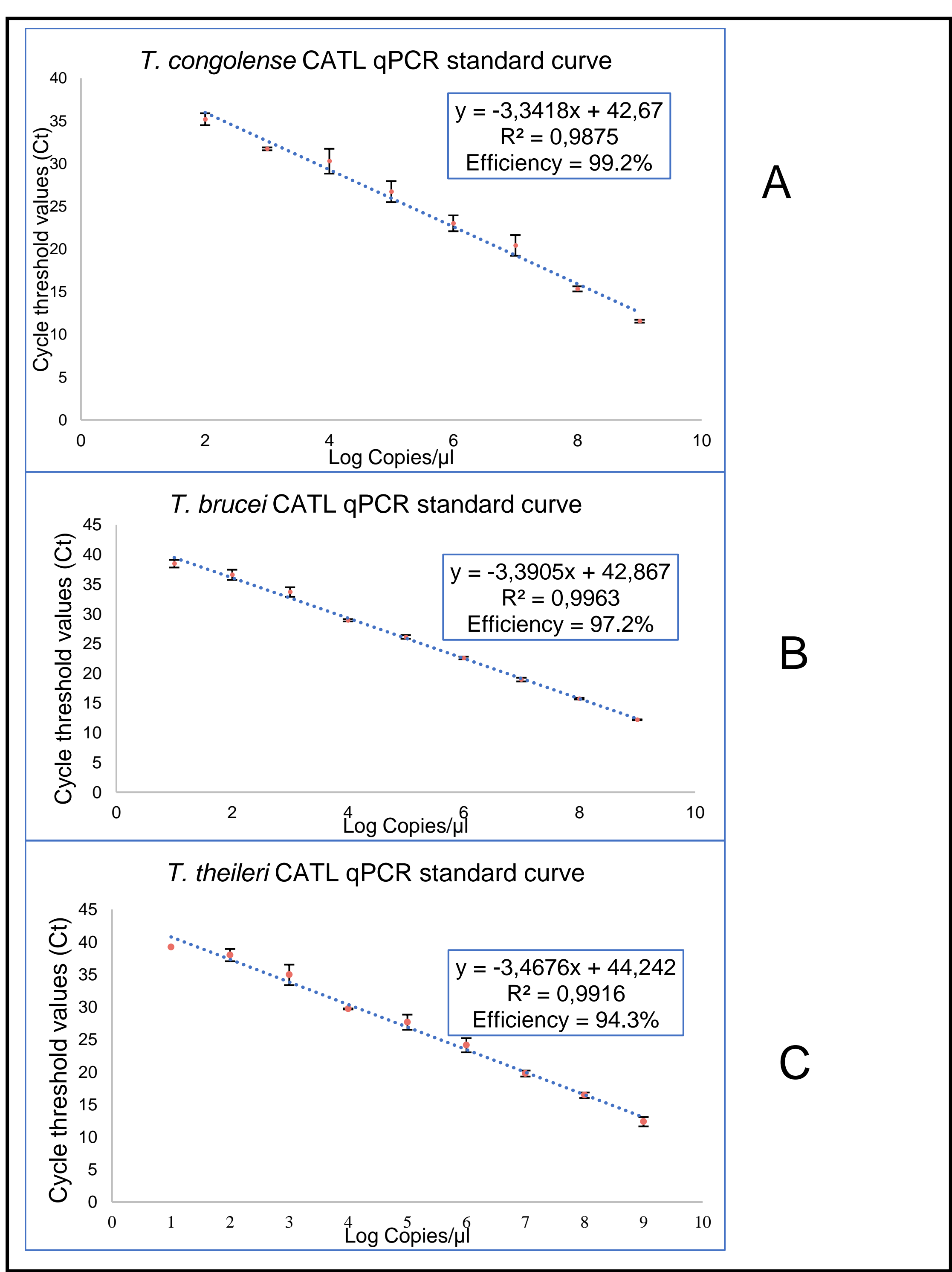


Figure 13: Linear regression for the quantification of A) *T. congolense* B) *T. brucei* C) *T. theileri* parasite DNA.
Cycle threshold (Ct) values were plotted against log copies/µl of the initial 10-fold dilution series of the parasite plasmid construct.

© University of Pretoria

3.3.4 Analytical specificity of the CATL qPCR assays

All three CATL qPCR assays were shown to be specific in the amplification of parasite DNA from either *T. congolense*, *T. brucei* or *T. theileri*. No amplification signals were observed from DNA isolated from other protozoal parasites known to infect cattle such as, *Babesia bovis*, *Babesia bigemina* and *Theileria parva*, other protozoal parasites known to infect cattle (Table 7). Cross-reactions between the assays were also not observed when each assay was tested against *Trypanosoma* DNA samples that included, *T. vivax*, *T. congolense*. *T. theileri* and *T. brucei* (Table 7).

Table 7: *Trypanosoma* CATL qPCR assays specificity.

Species name	*T. brucei*-specific qPCR assay Ct	*T. congolense*-specific qPCR assay Ct	*T. theileri*-specific qPCR assay Ct
B. bovis	negative	negative	negative
B. bigemina	negative	negative	negative
T. parva	negative	negative	negative
T. vivax	negative	negative	negative
T. brucei	26.21	negative	negative
T. congolense	negative	23.74	negative
T. theileri	negative	negative	25.71

3.3.5 Evaluation of cattle and goats' samples using the CATL qPCR assays

The developed CATL qPCR assays were tested for their ability to detect parasite DNA in field samples collected from both cattle and goats. Eight of the cattle samples (13.3%) tested positive for *T. congolense* with Ct values between 16.65 and 28.30 (mean 22.20, SD 3.84). Thirteen cattle field samples (21.7%) tested positive for *T. theileri* with Ct values between 30.71 and 37.78 (mean 35.43, SD 2.20). One cattle sample had mixed infections

© University of Pretoria

with *T. theileri* and *T. congolense*. *Trypanosoma brucei* infections were not detected in the cattle samples.

Only 10% (39 of 384) randomly selected goats' samples were screened for trypanosomes using the CATL qPCR assays. None of the samples tested positive for either *T. brucei* or *T. theileri*, but *T. congolense* parasite DNA could be detected in 9 (23.1%) samples using the *T. congolense* CATL qPCR assay. The Ct values for the *T. congolense* positive samples ranged between 30.41 and 35.99 (mean 34.18, SD 1.8).

3.4 Comparing diagnostics techniques for the detection of trypanosomes

Detection of trypanosomes in the 39 goats by the CATL qPCR assays was higher (9/39) that detected using either BCT or the ITS PCR assay. The CATL qPCR assays detected mixed infections in cattle, that were not detected by the other diagnostic techniques. Although CATL qPCR assays detected more infections in cattle than ITS qPCR assay (Table 8), Fisher's exact test showed the difference was not statistically significant (p=1.00). There was also no statistically significant difference between cattle infection rates detected by BCT or by ITS PCR assay (p=0.762). The qPCR assays detected significantly more cattle *Trypanosoma* infections than BCT and conventional PCR. Significantly higher cattle infection rates were detected by the CATL qPCR assays than by the ITS PCR assay (p=0.002) and BCT (p=0.014). Similarly, significantly higher cattle infection rates were detected by the ITS qPCR assay than by the ITS PCR (p=0.005) and BCT (p=0.023). Twelve of cattle samples were detected as *T. brucei* positive by the ITS qPCR. However, some of these *T. brucei* parasite DNA (66.7%), were subsequently identified as *T. theileri* by the *T. theileri* CATL qPCR assay. None of the *T. brucei* ITS qPCR positive samples were detected using the *T. brucei* CATL qPCR assay. The *T. congolense* CATL qPCR assay detected more *T. congolense* parasite DNA than BCT, ITS PCR assay and the ITS qPCR assay (Table 8).

© University of Pretoria

Table 8: Comparison of diagnostic techniques used in detecting trypanosomes.

	Cattle samples (n=60)				Goats' samples (n=39)		
	BCT	ITS PCR	ITS qPCR	CATL qPCR	BCT	ITS PCR	CATL qPCR
T. congolense	7	5	6	8		0	9
T. brucei		0	12	0		0	0
T. vivax		0	0	ND		0	0
T. theileri		ND	ND	13		ND	0
Total positive	7	5	18	21*	0	0	9
Negative	53	55	42	40	39	39	30
Significance		p=0.001				p=0.000	

*One sample had mixed infections of *T. congolense* and *T. theileri*.

*ND: Test was not done.

© University of Pretoria

CHAPTER 4

DISCUSSION

African animal trypanosomosis (AAT) is an incapacitating disease that impacts the health of cattle, and the livelihood of small-scale farmers in northern KwaZulu-Natal (NKZN). This is the only region in South Africa where AAT outbreaks have been recorded previously (Du Toit, 1954; Kappmeier *et al.*, 1998). African animal trypanosomosis remains an important disease that needs continuous monitoring to help improve control strategies and reduce economic damages (de Beer *et al.*, 2016). This study aimed at determining the occurrence of trypanosome infections in cattle and identifying the different *Trypanosoma* species circulating in cattle within the NKZN region. The study also aimed at determining the occurrence of *Trypanosoma* infections in goats, and therefore the possible role that goats might have in maintaining and spreading infections. The present study details the AAT results obtained from surveying 60 cattle and 384 goat samples collected at three diptanks within the NKZN region using both parasitological and molecular methods. The study further describes the development and application of a TaqMan minor groove binder (MGB™) real-time polymerase chain reaction (PCR) assay targeting the cathepsin-L (CATL) gene, for the quantitative detection and distinction between *Trypanosoma congolense*, *Trypanosoma brucei* and *Trypanosoma theileri* infections from cattle and goat blood samples.

4.1 Trypanosomes in cattle

4.1.1 Parasitological detection of trypanosomes in cattle

The buffy coat technique (BCT) was used as the parasitological diagnostic tool in this study due to its improved sensitivity and simplicity in detecting trypanosomes compared to other parasitological techniques (Paris *et al.*, 1982). The herd average packed cell volume (HA-PCV) and herd average anaemia (HAA) were used as AAT indicators, as well as, to indicate the health status and productivity of animals (Murray and Dexter, 1988; Van den Bossche and Rowlands, 2001). Animals with PCV of less than 24% were characterized anaemic. Buffy coat smears of samples collected from cattle in NKZN were used to determine the presence of trypanosomes at communal diptanks (Gillingwater *et al.*, 2010; Ntantiso *et al.*, 2014). Gillingwater *et al.* (2010) reported infection rates of 0.9%, 9.4% and 0% at the communal diptanks Ekuphindisweni, Mvutshini and Ocilwane, respectively. Subsequent

© University of Pretoria

studies done by Ntantiso *et al.* (2014) showed increased infection rates of 8.9%, 12.3% and 2.9%, respectively within these diptank areas due to the lack of control strategies. The current study shows that the infection rate is even higher using BCT at Ekuphindisweni (15%), Mvutshini (15%) and Ocilwane (5%) than reported by both Gillingwater *et al.* (2010) and Ntantiso *et al.* (2014). It was further observed that the HA-PCV of both infected and non-infected cattle were above the 24% cut-off, thus indicating that the cattle herds were in good health. In contrast, the HAA of infected cattle (57.1%) was significantly higher than the HAA (9.4%) observed for non-infected cattle, indicating that anaemia in infected cattle was a result of *Trypanosoma* infections. Some of the infected cattle had high PCV values, and were consequently non-anaemic, suggesting low parasitaemia in these positive cattle. Anaemia in the uninfected cattle suggests the presence of confounding factors such as poor nutrition or parasitic infestations (Van den Bossche and Rowlands, 2001). The observed HAA and HA-PCV results indicate that AAT detected in this study has an impact on the productivity of infected cattle.

Establishment of human settlements near game reserves and the herding of cattle in the proximity of these reserves, has increased game-livestock interactions (Simwango *et al.*, 2017). This proximity increases exposure of livestock to tsetse flies that transmit trypanosomes from wildlife reservoirs thus increasing the probability of *Trypanosoma* infections in livestock. The last extensive integrated control approach, which used treatment by ethidium bromide and dipping in a pyrethroid chemical, was done to control AAT after the 1990 outbreak in KwaZulu-Natal (KZN) (Bagnall, 1993; Kappmeier *et al.*, 1998). The lack of regulated control measures in these high tsetse challenged areas since the 1990 outbreak, continue to contribute to the increase of AAT in South Africa (Ntantiso *et al.*, 2014; de Beer *et al.*, 2016). In a study to monitor the productivity and health of cattle before and after AAT treatment, Latif *et al.* (2019), observed that cattle could get protection of up to 6 months after treatment against *Trypanosoma* infections. It was recommended that regular treatment (2-3 per year) of cattle together with integrated pest management of tsetse flies should be adopted for satisfactory results against AAT in NKZN (Latif *et al.*, 2019), since it is difficult to keep the livestock away from wildlife reservoirs and the tsetse challenge. Adoption of these control measures could substantially decrease AAT in South Africa.

4.1.2 Molecular detection of trypanosomes in cattle using conventional ITS PCR

Using conventional PCR assays targeting the 18S ribosomal ribonucleic acid (rRNA) gene and random deoxyribonucleic acid (DNA) fragments, high levels of trypanosome infections have been reported in cattle from NKZN, with *T. congolense* as the most abundant

© University of Pretoria

pathogenic trypanosome detected (Ledoka, 2008; Mamabolo *et al.*, 2009; Gillingwater *et al.*, 2010). The multiplex internal transcribed spacer 1 (ITS) polymerase chain reaction (PCR) described by Njiru *et al.* (2004a), was used in this study to identify the different *Trypanosoma* species circulating in cattle and goats in the NKZN region. This multiplex PCR was chosen as the preferred molecular assay as it reduces time and costs compared to 18S PCR- restriction fragment length polymorphism (RFLP) or species-specific PCRs, and it can also detect unexpected trypanosome species. *Trypanosoma* parasites, all of which were *T. congolense*, were detected in 8.3% of all cattle screened using the ITS PCR assay. Similar to previous studies, this study showed that the infection rate of *T. congolense* in cattle was higher at Mvutshini than at either Ekuphindisweni or Ocilwane diptanks (Ledoka, 2008; Gillingwater *et al.*, 2010). This result was expected as *Glossina austeni*, the main vector for *T. congolense* transmission in KwaZulu-Natal, has higher densities at Mvutshini than at Ekuphindisweni and Ocilwane (Motloang *et al.*, 2012; de Beer *et al.*, 2016).

All the ITS PCR positive cattle samples were also detected by the BCT. However, two of the seven cattle samples that tested positive for trypanosomes by BCT, did not test positive with PCR. A similar discrepancy was noted in a recent study by Matovu *et al.* (2020), which aimed at identifying haemoparasites circulating in a tsetse-challenged region in Uganda. In their study, several BCT positive samples were not detected by ITS PCR. They concluded that the discrepancy between the two assays might be due to the sensitivity of the ITS PCR assay which has been proven to be low when detecting infections of more than two *Trypanosoma* species (Njiru *et al.*, 2004a). Although the ITS PCR could detect *T. congolense* in this study, differentiation between the three subgroups of *T. congolense* (Savannah-type, Kilifi-type and Riverine/Forest-type) could not be determined. *Trypanosoma congolense* Savannah-type and Kilifi-type are the only subgroups that have been detected in NKZN and mixed infections by the two subgroups has been reported using type-specific PCR assays (Van den Bossche *et al.* 2006; Mamabolo *et al.*, 2009). The majority of trypanosome infections in cattle in NKZN are caused by virulent *T. congolense* Savannah-type, while the prevalence of the non-pathogenic *T. congolense* Kilifi-type is proportionally lower (Ledoka, 2008; Gillingwater *et al.*, 2010; Motloang, 2012). The possible presence of both *T. congolense* Savannah-type and Kilifi-type and/ or other trypanosomes in NKZN cattle samples could, therefore, limit detection by the ITS PCR.

© University of Pretoria

4.1.3 Trypanosome detection using ITS real-time PCR assay

The current study detected and differentiated trypanosome species circulating in cattle, in NKZN, using an ITS quantitative real-time PCR (qPCR) assay designed by Silbermayr *et al.* (2013) for the detection of *T. brucei*, *T. congolense* and *Trypanosoma vivax*. However, in this study each of the probes were labelled differently so that they could be used in a multiplex format.

Real-time PCR assays have better detection of trypanosomes in low levels of infection due to their high sensitivity, compared to parasitological techniques and conventional PCR assays (Taylor *et al.*, 2008; Sharma *et al.*, 2012; Silbermayr *et al.*, 2013). In a study conducted in India to determine the prevalence of *T. evansi* in cattle and buffaloes, both conventional parasitological and molecular methods of detection were compared (Sharma *et al.*, 2012). Their study showed that qPCR assay targeting the ITS1 rRNA gene was far more sensitive in detecting parasite DNA. Another study compared detection of *T. evansi* in experimentally infected mice using real-time PCR assay and the haematocrit centrifugation assay (Taylor *et al.*, 2008). The study showed the qPCR assay had better sensitivity and was faster in detecting trypanosomes (Taylor *et al.*, 2008). In the current study, the ITS qPCR assay detected *Trypanosoma* parasite DNA in 30% of the sixty cattle sampled. The ITS qPCR assay detected 61.1% and 72.2% *Trypanosoma* infections that were not detected by BCT and ITS PCR, respectively. These results are comparable to the findings by Silbermayr *et al.* (2013), who also observed that the ITS qPCR assay had better sensitivity and specificity than the ITS PCR.

Trypanosomes detected by the ITS qPCR assay, in the current study, were *T. congolense* and *T. brucei*. The identification of *T. brucei* as the most abundant trypanosome (20%) is very strange because it is a rare parasite in South Africa. *Trypanosoma brucei* had not been recorded in NKZN for several years (Van den Bossche *et al.*, 2006; Ledoka, 2008; Mamabolo *et al.*, 2009; Motloang *et al.*, 2012). However, Taioe, (2013), subsequently detected *T. brucei* in tsetse flies (*Glossina brevipalpis*) in the same region. The study used a nested PCR targeting the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene which identified 4 sequences with high homology to *T. brucei*. The author suggested that the detection of *T. brucei* indicates that there is a possibility that these parasites have remained undetected due to the lack of sensitivity of available diagnostic assays (Taioe, 2013). However, molecular analysis in prior studies only detected *T. congolense* in tsetse flies (Ledoka, 2008; Gillingwater *et al.*, 2010). It has been reported that the non-pathogenic parasite, *T. theileri* occurs globally and can sometimes confuse

© University of Pretoria

diagnostic techniques (Uilenberg, 1998; Desquesnes, 2004; Ngomtcho *et al.*, 2017). For these reasons, it was speculated that the *T. brucei* probe region in the ITS qPCR assay was cross-reacting with *T. theileri* DNA, resulting in the observed false-positive results. Consequently, the CATL TaqMan MGB™ qPCR assays were designed (section 4.2) to differentiate between *T. brucei*, *T. theileri* and *T. congolense* infections and to confirm whether the *T. brucei* positive samples detected were indeed false-positives.

Trypanosoma congolense parasite DNA could be detected in 10% of cattle sampled across all three diptanks. There was a strong correlation between the ITS PCR and ITS qPCR in the detection of *T. congolense*, as the qPCR assay detected all the *T. congolense* infections that were detected by the ITS PCR, with an additional *T. congolense* positive sample detected at Ekuphindisweni.

Trypanosoma vivax, together with *T. congolense*, were responsible for the 1990 AAT outbreak in NKZN which killed over 10 000 cattle (Kappmeier *et al.*, 1998). Subsequent prevalence studies reported these two trypanosomes as the major AAT causative parasites, with *T. vivax* accounting to very few infections (Van den Bossche *et al.*, 2006; Ledoka, 2008; Mamabolo *et al.*, 2009). Studies that followed were, therefore, not inclusive of *T. vivax* and only reported prevalence of *T. congolense* (Gillingwater *et al.*, 2010; Ntantiso *et al.*, 2014; Latif *et al.*, 2019). The current study did not detect any *T. vivax* infection by both the conventional ITS PCR and the qPCR assays. A study that was conducted in NKZN to determine the *Trypanosoma* prevalence in the area, also did not detect *T. vivax* infections in cattle using a PCR targeting the ITS regions (Taioe, 2013). However, a much larger sampling size, that is inclusive of game animals, bovines and small ruminants, is needed to understand this parasite's potentially new distribution pattern or the possibility of its absence in the region.

4.2 Development of the CATL qPCR assays

To improve the detection and differentiation of *Trypanosoma* species infecting livestock (AAT), a molecular assay targeting the CATL gene, which has been reported as a suitable marker for species discrimination, was developed (Cortez *et al.*, 2009; Rodrigues *et al.*, 2010). The CATL-like gene has been previously targeted, in PCR assays, to detect *T. vivax* (Cortez *et al.*, 2009), *Trypanosoma rangeli* (Ortiz *et al.*, 2009) and *T. theileri* (Rodrigues *et al.*, 2010).

The developed qPCR assays based on CATL for each species showed excellent linearity and high specificity, and that there was no cross-reactivity between *Trypanosoma* species

© University of Pretoria

tested. The detection limits ranged from 10^9 to $10^2/10^1$ copies/µl and the efficiencies ranged from 94-100%. *In silico* BLAST analysis of each of species-specific probe sequences indicated 100% sequence homology to *Trypanosoma*. The *T. congolense* probe sequence showed 100% sequence identity to published *T. congolense* sequences on GenBank. The *T. brucei* CATL probe sequence, however, showed 100% sequence identity to *Trypanosoma evansi* and *Trypanosoma equiperdum* CATL sequences. This is not surprising as *T. evansi*, *T. brucei* and *T. equiperdum* belong to the *Trypanozoon* subgenus. The *T. brucei* CATL qPCR assay could potentially be used in the detection of parasites belonging to the *Trypanozoon* subgenus. The *T. theileri* CATL probe sequence also showed 100% sequence identity to *Trypanosoma* (*Megatrypanum*) *trinaperronei*, a parasite characterized in white-tailed deer, which was shown to be phylogenetically related to *T. theileri* (Garcia *et al.*, 2020).

Trypanosome research in South Africa has not focused much on *T. theileri* as it is a non-pathogenic parasite. However, *T. theileri* poses as a risk when the health status of the herd is compromised by other confounding factors such as malnutrition and co-infections with other parasites (Doherty *et al.*, 1993; Seifi, 1995). Accurate diagnosis of the trypanosomes is crucial for epidemiological studies.

4.2.1 Detection of trypanosomes in cattle using CATL qPCR assays

An evaluation of 60 cattle samples showed that the animals were infected with *T. congolense*, and *T. theileri* parasites. In addition, the CATL qPCR assays could detect parasite DNA in 24.5% of samples that were BCT negative. Confirming that qPCR is far more sensitive than BCT. The developed CATL qPCR assays detected 3 more *Trypanosoma* infections in the 60 cattle samples than the ITS qPCR. All the ITS PCR and ITS qPCR *T. congolense* positive samples were confirmed by the *T. congolense* CATL qPCR assay. Thirteen (21.7%) of the cattle field samples tested positive for *T. theileri* with Ct values between ranging between 30.7 and 38.32 (Ct≥39 regarded as negative). Two of these samples were detected at Ct values above 37.97 (10^2 copies/µl) indicating very low parasitaemias and possibly false positives.

In contrast to the ITS qPCR assay which detected *T. brucei* infections, the *T. brucei* CATL qPCR assay did not detect any *T. brucei* infections in the cattle samples. Most of the samples (66.7%) that were identified as *T. brucei* positive by ITS qPCR assay, were identified as *T. theileri* positive by the CATL qPCR assays. These findings provide evidence to the earlier hypothesis that there is a cross-reaction, potentially between the ITS qPCR *T. brucei* probe and *T. theileri* DNA sequences, which should be further investigated. The

© University of Pretoria

remaining 33.3% of the *T. brucei* positive samples (by ITS qPCR) were not identified by the CATL qPCR assays. A study on AAT prevalence in KZN, reported the occurrence of both *T. brucei* and *T. theileri* (Taioe, 2013). In the current study, however, the *T. brucei* positive samples need to be further cloned, sequenced and confirmed with more definitive diagnostics tools.

The study by Taioe, (2013) also reported the occurrence of *T. congolense* and *T. theileri* as single infections in cattle. In this study, the developed CATL qPCR assays confirmed the presence of *T. theileri* in NKZN. However, in contrast to the findings reported by Taioe, (2013), the CATL qPCR assays detected mixed infections with two parasites. Although the current study documented *T. congolense* as the most abundant pathogenic trypanosome, the non-pathogenic *T. theileri* was the overall most abundant parasite. Application of highly sensitive and specific tools, such as the CATL qPCR assays, in AAT epidemiological studies might reveal much higher *T. theileri* and *T. congolense* prevalence rates in NKZN. It is possible that the prevalence of AAT in South Africa might have been under-reported in previous years, as the diagnostics tools that have been used (such as BCT and conventional PCR) are of less sensitivity than the real-time PCR tool.

All the diagnostic techniques used in the current study observed that there were less *Trypanosoma* positive cattle at Ocilwane diptank than at Ekuphindisweni and at Mvutshini. The results from this study are similar to the findings from Ntantiso *et al.* (2014), which show a lower *Trypanosoma* prevalence at Ocilwane than other diptanks. Ocilwane has a low prevalence due to its geographic location, which is at the southern part of NKZN, where there is a lower population of tsetse flies (Ntantiso *et al.*, 2014; de Beer *et al.*, 2016). One *Trypanosoma* positive reading by BCT was not detected by all the molecular assays used in this study. It is very well established that molecular assays have higher sensitivity than parasitological techniques, including the BCT (Van Den Bossche *et al.*, 2006; Marcotty *et al.*, 2008; Gillingwater *et al.*, 2010; N'Djetchi *et al.*, 2017). However, some studies have reported better detection by microscopy, compared to molecular assays (Mattioli and Faye, 1996; Junqueira *et al.*, 1996; Njiru *et al.*, 2004b; Moti *et al.*, 2014; Matovu *et al.*, 2020). This could be associated with poor DNA quality, the presence of inhibitors or the inadequate optimization of the molecular assay (Wolk *et al.*, 2001). The results from this study shows that *Trypanosoma* infection rates within the NKZN have increased since the last epidemiological surveys (Van den Bossche *et al.*, 2006; Ledoka, 2008; Mamabolo *et al.*, 2009, Taioe, 2013; de Beer *et al.*, 2016), thus suggesting that more regulated intervention against the disease is required.

© University of Pretoria

4.3 Detection of trypanosomes in goats

The detection of *Trypanosoma* species in goats using parasitological techniques have been reported in Zambia, Nigeria and Cote d'Ivore (Bealby *et al.*, 1996; Chinyere and Juliet, 2013; N'Djetchi *et al.*, 2017). Buffy coat technique was used to determine natural infection in goats by *T. vivax*, *T. congolense* and *T. brucei* in Zambia, and *T. vivax* and *T. brucei* in Nigeria (Bealby *et al.*, 1996; Chinyere and Juliet, 2013). Bealby and Connor, (1996) showed that *Trypanosoma* infections significantly affected body weight, fertility, lactation, growth and mortality in goats. However, they also showed goats born in high tsetse challenge areas acquired some degree of trypanotolerance and had better survival rates than goats taken from tsetse-free areas and exposed to high tsetse challenge. *Trypanosoma congolense*, *T. vivax*, *T. simiae* and *T. brucei* parasite DNA have been reported using PCR assays in goats across Africa. In Zambia, Nyimba *et al.* (2015) reported 23.7% overall prevalence by *T. congolense*, *T. brucei* and *T. vivax*. In a study in Côte d'Ivoire, 6.6% of goats were positive for *T. congolense*, *T. vivax* and *T. brucei* infections by PCR (N'Djetchi *et al.*, 2017). Studies in The Gambia have detected *T. vivax*, *T. brucei* and *T. congolense* using PCR assays (Pereira de Almeida *et al.*, 1998; Osaer *et al.*, 1999). In Kenya, *T. vivax*, *T. simiae*, *T. congolense* and *Trypanosoma brucei rhodesiense* were detected in goats by PCR (Ng'ayo *et al.*, 2005). However, in the present study, there were no trypanosome infections detected by both BCT and ITS PCR, in all the 384 goats. Although the study by Taioe, (2013) reported the detection of trypanosomes in both cattle and tsetse flies, the detection of any *Trypanosoma* infections in goats using a PCR assay targeting the ITS gene was consistently negative. In a study done by Simukoko *et al.* (2007), to determine the significance of goats, cattle and pigs in AAT epidemiology on the eastern plateau of eastern Zambia, no AAT infection was found in goats by parasitological analysis. However, 3.3% AAT prevalence was determined in goats using the molecular analysis, with *T. congolense* being the predominant species. They concluded that molecular analysis was a better diagnostic tool in detecting trypanosomes. Simukoko *et al.* (2007), also observed that when the majority of livestock present in an area is cattle, then tsetse flies will be more attracted to feed on cattle than other small ruminants. Thus, infections rates are likely to be higher in cattle than small ruminants such as goats.

4.3.1 Detection of trypanosomes in goats using CATL qPCR assays

Research on AAT occurrence and prevalence in goats in NKZN is limited, however, a study by Ng'ayo *et al.* (2005) on natural *Trypanosoma* infections in small ruminants have shown that goats can be potential reservoirs of infections to other domesticated animals. In the

© University of Pretoria

present study, the BCT and the ITS PCR assay failed to identify trypanosome infections in goats. However, screening using the CATL qPCR assays detected *T. congolense* infections in 9 of 39 goat samples. None of the goats tested positive for *T. theileri* and/or *T. brucei*. The current study reports the first time on the molecular diagnosis of *Trypanosoma* infections in goats in NKZN. The identification of 23.1% of *T. congolense* infections could suggest that goats have a potential role to play in the maintenance and spread of *Trypanosoma* infections. Highly sensitive tools such as the CATL qPCR assays should, therefore, be applied to further assess trypanosome infections in goats using a much larger sample size.

The detection of *Trypanosoma* infections in goats using qPCR assays indicates the superior sensitivity of qPCR over conventional parasitological techniques and conventional PCR assays. The observed high Ct values are indicative of low parasitaemia, which explains why these parasites could not be detected using BCT and ITS PCR. Low parasitaemia of trypanosomes in goats was observed in a molecular diagnostics study in Zambia (Musinguzi *et al.*, 2016). The study used PCR assays that targeted the ITS1 gene and the CATL gene of *T. vivax* isolates, and recorded no significant differences in the PCV values of the *Trypanosoma* infected and non-infected goats. It was concluded that AAT in goats does not usually show clinical signs (Musinguzi *et al.*, 2016).

Goats from the three areas sampled, freely graze closer to Hluhluwe-uMfolozi game reserve which increases interactions with tsetse flies. In spite of this exposure, *Trypanosoma* infections in goats have been shown to be less virulent than in cattle (Mahmoud and Elmalik, 1977). The study by Mahmoud and Elmalik, (1977) showed goats, experimentally infected with *T. congolense*, recovered from chronic infections, yet calves experimentally infected with the same *T. congolense*, died from trypanosomosis infections. However, the presence of trypanosomes should not be overlooked since trypanosomes coupled together with other constraining factors such as poor nutrition, helminth infections, can cause serious chronic infections in goats.

The probability of more *Trypanosoma* species circulating in goats in NKZN cannot be ruled out as only a few goats' samples were screened by the qPCR. Several studies have shown goats can be naturally infected with *T. brucei* (Osaer *et al.*, 1999; Nyimba *et al.*, 2015; N'Djetchi *et al.*, 2017), *Trypanosoma cruzi* (Herrera *et al.,* 2005), *T. vivax* (Pereira de Almeida *et al.*, 1998; N'Djetchi *et al.*, 2017), *T. evansi* (Boid *et al.*, 1981; Pereira de Almeida *et al.*, 1998), *Trypanosoma theodori* and *Trypanosoma uniforme* (Hoare, 1972; Smith and

© University of Pretoria

Sherman, 1994). In NKZN, goats are likely to be naturally infected with *T. congolense*, *T. vivax* and possibly *T. brucei* as these are the major trypanosomes reported in the area.

© University of Pretoria

CHAPTER 5

CONCLUSIONS

Findings from the molecular and parasitological survey of African animal trypanosomosis (AAT) in goats and cattle showed the pathogenic *Trypanosoma congolense* is prevalent at the three diptanks sampled in northern KwaZulu-Natal (NKZN). This finding confirms the results of previous studies that observed *T. congolense* as the major pathogenic trypanosome in the region. Although *Trypanosoma brucei* was reported in the survey, these samples were considered suspect positive samples due to their low parasitaemia and scarcity of the parasite in NKZN. *Trypanosoma vivax* was not detected in this study, which confirms its low occurrence in the region.

Comparison of the three diagnostic tools used in the survey revealed that there was no significant difference in infection rates detected by both the internal transcribed spacer (ITS) polymerase chain reaction (PCR) and by the buffy coat technique (BCT). However, the ITS quantitative real-time PCR (qPCR) assay proved to be a better diagnostic tool in detecting trypanosomes than the ITS PCR and BCT, showing higher sensitivity of real-time PCR assays.

The three TaqMan minor groove binder (MGB™) qPCR assays that were designed to target the cathepsin L-like (CATL) gene of *T. congolense*, *Trypanosoma theileri* and *T. brucei*, successfully amplified DNA of their respective target trypanosomes. The assays also reported the first molecular diagnosis of *T. congolense* in goats, in South Africa. This finding suggests that goats contribute in maintaining and spreading *Trypanosoma* infections to other livestock. The assays further illustrated the presence of the non-pathogenic *T. theileri* in most of the cattle samples that were previously described as *T. brucei* positive by the ITS qPCR. However, the CATL qPCR assays were unable to detect a few *T. brucei* ITS qPCR positive field samples. Further work should, therefore, be done for a definitive diagnosis of these suspect positive samples.

The CATL qPCR assays showed better detection of trypanosomes in the cattle and goats' field samples, than the ITS qPCR assay, ITS PCR and BCT. However, further work is required in order to validate the assays for diagnostic purposes, and it would also be interesting to evaluate the CATL qPCR assays in duplex or multiplex format. Highly

© University of Pretoria

sensitive diagnostics tools, such as real-time PCR assays, should be used in AAT studies for better understanding of the disease status, and should be used to gather deeper knowledge on T*rypanosoma* diversity in South Africa. Integrated control strategies that also target trypanosomes in small ruminants should be implemented to control AAT in NKZN.

© University of Pretoria

REFERENCES

ABD-ALLA, A.M. BERGOIN, M. PARKER, A.G. MANIANIA, N.K. VLAK, J.M. BOURTZIS, K. BOUCIAS, D.G. & AKSOY, S. 2013. Improving sterile insect technique (SIT) for tsetse flies through research on their symbionts and pathogens. *Journal of Invertebrate Pathology*, 112: S2-S10.

ABDI, R.D. AGGA, G.E. AREGAWI, W.G. BEKANA, M. VAN LEEUWEN, T. DELESPAUX, V. & DUCHATEAU, L. 2017. A systematic review and meta-analysis of trypanosome prevalence in tsetse flies. *BMC Veterinary Research*, 13(1): 100-130.

AHMED, H.A. MACLEOD, E.T. WELBURN, S.C. & PICOZZI, K. 2015. Development of real time PCR to study experimental mixed infections of *T. congolense* Savannah and *T. b. brucei* in *Glossina morsitans morsitans*. *PLOS ONE*, 10(3): e0117147.

ALSAN, M. 2015. The effect of the tsetse fly on African development. *American Economic Review*, 105: 382-410.

ALTSCHUL, S.F. GISH, W. MILLER, W. MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215(3): 403-410.

ASSEFA, S. & SHIBESHI, W. 2018. Drug resistance in African animal trypanosomes: A review. *African Journal of Microbiology Research*, 12(17): 380-386.

AUTY, H. TORR, S. MICHOEL, T. JAYARAMAN, S. & MORRISON, L. 2015. Cattle trypanosomosis: the diversity of trypanosomes and implications for disease epidemiology and control. *Revue Scientifique et Technique de l'OIE*, 34: 587-598.

AZAB, M.E. & ABDEL-MAKSOUD, H.A. 1999. Changes in some hematological and biochemical parameters during prepartum and postpartum periods in female Baladi goats. *Small Ruminant Research*, 34(1): 77-85.

BAGNALL, R.J. 1993. Trypanosomosis in Zululand. *Proceedings of the 24th Meeting of the SARCCUS Standing Committee for Animal Health,* Mbabane, Swaziland, pp. 41-49.

BAKER, J.R. 1995. The subspecific taxonomy of *Trypanosoma brucei*. *Parasite*, 2(1): 3-12.

© University of Pretoria

BARAL, T.N. 2010. Immunobiology of African trypanosomes: Need of alternative interventions. *Journal of Biomedicine and Biotechnology*, 2010: 1-24.

BEALBY, K.A. CONNOR, R.J. & ROWLANDS, G.J. 1996. Trypanosomosis in goats in Zambia. Nairobi, Kenya: International Livestock Research Institute.

BECKER, S. FRANCO, J. SIMARRO, P. STICH, A. ABEL, P. & STEVERDING, D. 2004. Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. *Diagnostic Microbiology and Infectious Disease*, 50(3): 193-199.

BENGALY, Z. SIDIBE, I. GANABA, R. DESQUESNES, M. BOLY, H. & SAWADOGO, L. 2002. Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. *Veterinary Parasitology*, 108(1): 1-19.

BOID, R. EL AMIN, E.A. & MAHMOUD, M.M. & LUCKINS, A.G. 1981. *Trypanosoma evansi* infections and antibodies in goats, sheep and camels in the Sudan. *Tropical Animal Health and Production*, 13(1): 141-146.

BONO BATTISTONI, M.F. ORCELLET, V. PERALTA, J.L. MARENGO, R. PLAZA, D. BRUNINI, A. RUIZ, M. WIDENHORN, N. SANCHEZ, A. MONJE, L. & CIGNETTI, L. 2016. First report of *Trypanosoma evansi* in a canine in Argentina. *Veterinary Parasitology: Regional Studies and Reports*, 6: 1-3.

BROWN, K. 2008. From Ubombo to Mkhuzi: Disease, colonial science, and the control of nagana (livestock trypanosomosis) in Zululand, South Africa, c. 1894-1953. *Journal of the History of Medicine and Allied Sciences*, 63: 285-322.

CHAMOND, N. COSSON, A. BLOM-POTAR, M.C. JOUVION, G. D'ARCHIVIO, S. MEDINA, M. DROIN-BERGERE, S. HUERRE, M. GOYARD, S. & MINOPRIO, P. 2010. *Trypanosoma vivax* infections: Pushing ahead with mouse models for the study of nagana. I. parasitological, hematological and pathological parameters. *PLOS Neglected Tropical Diseases,* 4: e792.

CHINYERE, A.L. & JULIET, O.O. 2013. Trypanosomiasis in red sokoto and West African dwarf goats at Ikpa Abattoir, Nsukka, Enugu State, Nigeria. *Journal of Entomology and Zoology Studies*, 1: 35-37.

© University of Pretoria

CHITANGA, S. MARCOTTY, T. NAMANGALA, B. VAN DEN BOSSCHE, P. VAN DEN ABBEELE, J. & DELESPAUX, V. 2011. High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. *PLOS Neglected Tropical Diseases*, 5(12): e1454.

CONNOR, R.J. 1994. The impact of nagana. *Onderstepoort Journal of Veterinary Research*, 61(4): 379-383.

CONNOR, R.J. & VAN DEN BOSSCHE, P. 2004. African animal trypanosomoses. In Coetzer, J.A.W. & Tustin R.C. (Eds.), *Infectious diseases of livestock*. 2nd edition. Cape town: Oxford University Press, pp. 251-296.

COOK, G.C. 1994. Sir David Bruce's elucidation of the aetiology of nagana-exactly one hundred years ago. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88: 257-258.

CORTEZ, A. RODRIGUES, A.C. GARCIA, H.A. NEVES, L. BATISTA, J.S. BENGALY, Z. PAIVA, F. & TEIXEIRA, M.M.G. 2009. Cathepsin L-like genes of *Trypanosoma vivax* from Africa and South America - characterization, relationships and diagnostic implications. *Molecular and Cellular Probes*, 23: 44-51.

CORTEZ, A.P. VENTURA, R.M. RODRIGUES, A.C. BATISTA, J.S. PAIVA, F. AÑEZ, N. MACHADO, R.Z. GIBSON, W.C. & TEIXEIRA, M.M.G. 2006. The taxonomic and phylogenetic relationships of *Trypanosoma vivax* from South America and Africa. *Parasitology*, 133: 159-169.

COURTIN, D. BERTHIER, D. THEVENON, S. DAYO, G. GARCIA, A. & BUCHETON, B. 2008. Host genetics in African trypanosomiasis. *Infection, Genetics and Evolution*, 8: 229-238.

COX, A. TILLEY, A. MCODIMBA, F. FYFE, J. EISLER, M.C. HIDE, G. & WELBURN, S.C. 2005. A PCR based assay for detection and differentiation of African trypanosome species in blood. *Experimental Parasitology*, 111: 24-29.

CUMMINGS, K. & TARLETON, R. 2003. Rapid quantitation of *Trypanosoma cruzi* in host tissue by real-time PCR. *Molecular and Biochemical Parasitology*, 129(1): 53-59.

© University of Pretoria

DARJI, A. LUCAS, R. MAGEZ, S. TORREELE, E. PALACIOS, J. SILEGHEM, M. BAJYANA SONGA, E. HAMERS, R. & DE BAETSELIER, P. 1992. Mechanisms underlying trypanosome-elicited immunosuppression. *Annales de la Société Belge de Médecine Tropicale*, 72(Suppl 1): 27-38.

DE BEER, C. VENTER, G. KAPPMEIER GREEN, K. ESTERHUIZEN, J. DE KLERK, D. NTSHANGASE, J. VREYSEN, M. PIENAAR, R. MOTLOANG, M. NTANTISO, L. & LATIF, A. 2016. An update of the tsetse fly (Diptera: Glossinidae) distribution and African animal trypanosomosis prevalence in north-eastern KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, 83(1): a1172.

DELESPAUX, V. AYRAL, F. GEYSEN, D. & GEERTS, S. 2003. PCR-RFLP using Ssu-rDNA amplification: Applicability for the diagnosis of mixed infections with different trypanosome species in cattle. *Veterinary Parasitology*, 117: 185-193.

DELESPAUX, V. GEYSEN, D. VAN DEN BOSSCHE, P. & GEERTS, S. 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. *Trends in Parasitology*, 24: 236-242.

DESQUESNES, M. 2004. Livestock trypanosomoses and their vectors in Latin America. *CIRAD-EMVT publication,* OIE, Paris, France, pp. 11-20.

DESQUESNES, M. & DÁVILA, A.M. 2002. Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Veterinary Parasitology*, 109(3-4):213-31.

DESQUESNES, M. & DIA, M. 2003. Mechanical transmission of *Trypanosoma congolense* in cattle by the African tabanid *Atylotus agrestis*. *Experimental Parasitology*, 105: 226-231.

DESQUESNES, M. HOLZMÜLLER, P. LAI, D.H. DARGANTES, A. LUN, Z.R. & JITTAPLAPONG, S. 2013. *Trypanosoma evansi* and surra: a review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. *BioMed Research International*, 2013: 194176.

DESQUESNES, M. MCLAUGHLIN, G. ZOUNGRANA, A. & DÁVILA, A. 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *International Journal for Parasitology*, 31(5-6): 610-614.

© University of Pretoria

DOHERTY, M.L. WINDLE, H. VOORHEIS, H.P. LARKIN, H. CASEY, M. CLERY, D. & MURRAY, M. 1993. Clinical disease associated with *Trypanosoma theileri* infections in a calf in Ireland. *Veterinary Record*, 26: 653-656.

DU TOIT, R. 1954. Trypanosomiasis in Zululand and the control of tsetse flies by chemical means. *Onderstepoort Journal of Veterinary Research*, 26: 317-387.

DYER, N. ROSE, C. EJEH, N. & ACOSTA-SERRANO, A. 2013. Flying tryps: survival and maturation of trypanosomes in tsetse flies. *Trends in Parasitology*, 29: 188-196.

EBERHARDT, A.T. MONJE, L.D. ZURVERA, D.A. & BELDOMENICO, P.M. 2014. Detection of *Trypanosoma evansi* infection in wild capybaras from Argentina using smear microscopy and real-time PCR assays. *Veterinary Parasitology*, 202: 226-233.

EBHODAGHE, F. ISAAC, C. & OHIOLEI, J. 2018. A meta-analysis of the prevalence of bovine trypanosomiasis in some African countries from 2000 to 2018. *Preventive Veterinary Medicine*, 160: 35-46.

EDGAR, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5): 1792-1797.

EMSLIE, F. 2004. A field evaluation of three trypanosomosis control strategies, in KwaZulu-Natal, South Africa. MSc Dissertation. University of Pretoria, South Africa.

ESTERHUIZEN, J. KAPPMEIER GREEN, K. MARCOTTY, T. & VAN DEN BOSSCHE, P. 2005. Abundance and distribution of the tsetse flies, *Glossina austeni* and *G. brevipalpis*, in different habitats in South Africa. *Medical and Veterinary Entomology*, 19: 367-371.

ESTERHUIZEN, J. & VAN DEN BOSSCHE, P. 2006. Protective netting, an additional method for the integrated control of livestock trypanosomosis in KwaZulu-Natal Province, South Africa. *Onderstepoort Journal of Veterinary Research*, 73: 9-321.

EYFORD, B. SAKURAI, T. SMITH, D. LOVELESS, B. HERTZ-FOWLER, C. DONELSON, J. INOUE, N. & PEARSON, T. 2011. Differential protein expression throughout the life cycle of *Trypanosoma congolense*, a major parasite of cattle in Africa. *Molecular and Biochemical Parasitology*, 177: 116-125.

© University of Pretoria

EYSSEN, L.E. VATHER, P. JACKSON, L. XIMBA, P. BITEAU, N. BALTZ, T. BOULANGÉ, A. BÜSCHER, P. & COETZER, T.H.T. 2018. Recombinant and native TviCATL from *Trypanosoma vivax*: Enzymatic characterisation and evaluation as a diagnostic target for animal African trypanosomosis. *Molecular and Biochemical Parasitology*, 223: 50-54.

FIRESBHAT, A. & DESALEGN, C. 2015. Epidemiology and impacts of trypanosomiasis in cattle. *European Journal of Applied Sciences*, 7(5): 220-225.

FREITAS, J.M. LAGES-SILVA, E. CREMA, E. PENA, S.D. & MACEDO, A.M. 2005. Real time PCR strategy for the identification of major lineages of *Trypanosoma cruzi* directly in chronically infected human tissues. *International Journal for Parasitology*, 35(4): 411-417.

GARDINER, P.R. & MAHMOUD, M.M. 1992. Salivarian trypanosomes causing disease in livestock outside sub-saharan Africa. In Kreier, J.P. & Baker, J.R. (Eds.), *Parasitic Protozoa*. 2nd edition. London: Academic Press, pp. 277-314.

GARCIA, H.A. BLANCO, P.A. RODRIGUES, A.C. RODRIGUES, C.M.F. TAKATA, C.S.A. CAMPANER, M. CAMARGO, E.P. & TEIXEIRA, M.M.G. 2020. Pan-American *Trypanosoma* (*Megatrypanum*) *trinaperronei* n. sp. in the white-tailed deer *Odocoileus virginianus* Zimmermann and its deer ked *Lipoptena mazamae* Rondani, 1878: morphological, developmental and phylogeographical characterisation. *Parasites Vectors*, 13: 308-325.

GEYSEN, D. DELESPAUX, V. & GEERTS, S. 2003. PCR–RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. *Veterinary Parasitology*, 110(3-4): 171-180.

GIBSON, W. & BAILEY, M. 2003. The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes. *Kinetoplastid biology and disease*, 2(1): 1-13.

GIBSON, W. KAY, C. & PEACOCK, L. 2017. *Trypanosoma congolense*: Molecular toolkit and resources for studying a major livestock pathogen and model trypanosome. *Advances in Parasitology*, 98: 283-309.

GIBSON, W. STEVENS, J. & TRUC, P. 1999. Identification of trypanosomes: from morphology to molecular biology, In Dumas, M. Bouteille, B. & Buguet, A. (Eds.), *Progress in human African trypanosomiasis, sleeping sickness*. Paris: Springer Verlag, pp. 7-29.

© University of Pretoria

GILLINGWATER, K. MAMABOLO, M. & MAJIWA, P. 2010. Prevalence of mixed *Trypanosoma congolense* infections in livestock and tsetse in KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association*, 81(4): 219-223.

GIORDANI, F. MORRISON, L.J. ROWAN, T.G. DE KONING, H.P. & BARRETT, M.P. 2016. The animal trypanosomiases and their chemotherapy: a review. *Parasitology*, 143(14): 1862-1889.

GRIFFIN, L. 1978. African trypanosomosis in sheep and goats: a review. *Veterinary Bulletin*, 48: 819-825.

GRIFFIN, L. & ALLONBY, E. 1979. Disease syndromes in sheep and goats naturally infected with *Trypanosoma congolense*. *Journal of Comparative Pathology*, 89: 457-464.

GUTIERREZ, C. CORBERA, J.A. MORALES, M. & BÜSCHER, P. 2006. Trypanosomosis in goats: current status. *Annals of the New York Academy of Sciences*, 1081: 300-310.

HARRIS, R.H.T.P. 1932. Some facts and figures regarding the attempted control of *Glossina pallidipes* in Zululand. *South African Journal of Science*, 29: 495-507.

HENDRICKX, G. 2002. Tsetse presence-absence prediction model for *Glossina austeni* and *Glossina brevipalpis* in KwaZulu-Natal - South Africa. *Consultant report prepared by Agriculture and Veterinary Intelligence and Analysis (AVIA-GIS) to the International Atomic Energy Agency,* IAEA, Vienna, Austria.

HENDY, C.R.C. 1988. The effects of trypanosomosis prophylaxys and antihelminthic treatment in goats under traditional management in southern Tanzania. *Proceedings of the 1987 Livestock Production in Tsetse Affected Areas of Africa*, ILCA/ILRAD, Nairobi, Kenya, pp. 289-309.

HERRERA, L. D'ANDREA, P.S. XAVIER, S.C.C. MANGIA, R.H. FERNANDES, O. & JANSEN, A.M. 2005. *Trypanosoma cruzi* infection in wild mammals of the National Park 'Serra da Capivara' and its surroundings (Piauí, Brazil), an area endemic for Chagas disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 99(5): 379-388.

HOARE, C.A. 1966. The classification of mammalian trypanosomes. *Ergebnisse der Mikrobiologie, Immunitatsforschung und Experimentellen Therapie*, 39: 43-57.

© University of Pretoria

HOARE, C. 1972. The trypanosomes of mammals, a zoological monograph. Oxford, England: Blackwell Scientific Publications.

HOLMES, P. 2013. Tsetse-transmitted trypanosomes - Their biology, disease impact and control. *Journal of Invertebrate Pathology*, 112: S11-S14.

IRUNGU, P. NYAMWARO, S.O. & MASIGA, D.K. 2002. Financial implication of rearing sheep and goats under natural trypanosomosis challenge at Galana ranch Kenya. *Tropical Animal Health and Production*, 34: 503-513.

JONES, T.W. & DAVILA, A.M. 2001. *Trypanosoma vivax:* out of Africa. *Trends in Parasitology*, 17(2): 99-101.

JUNQUEIRA, A. CHIARI, E. & WHICKER, P. 1996. Comparison of the polymerase chain reaction with two classical parasitological methods for the diagnosis of Chagas disease in an endemic region of north-eastern Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90: 129-132.

KALTENBOECK, B. & WANG, C. 2005. Advances in real-time PCR: application to clinical laboratory diagnostics. *Advances in clinical chemistry*, 40: 219-259.

KAPPMEIER GREEN, K. 2002. Strategy for monitoring and sustainable integrated control or eradication of *Glossina brevipalpis* and *G. austeni* (Diptera: *Glossinidae*) in South Africa. PhD Thesis. University of Pretoria, South Africa.

KAPPMEIER, K. 2000. A newly developed odour-baited “H trap” for the live collection of *Glossina brevipalpis* and *Glossina austeni* (Diptera: Glossinidae) in South Africa. *Onderstepoort Journal of Veterinary Research*, 67: 15-26.

KAPPMEIER, K. NEVILL, E. & BAGNALL, J. 1998. Review of tsetse flies and trypanosomosis in South Africa. *Onderstepoort Journal of Veterinary Research*, 65: 195-203.

KONNAI, S. MEKATA, H. MINGALA, C. N. ABES, N. S. GUTIERREZ, C. A. HERRERA, J. R. DARGANTES, A. P. WITOLA, W. H. CRUZ, L. C. & INOUE, N. 2009. Development and application of a quantitative real-time PCR for the diagnosis of surra in water buffaloes. *Infection, Genetics and Evolution*, 9: 449-452.

© University of Pretoria

KRINSKY, W.L. 2002. Tsetse flies (Glossinidae). In Mullen, G. & Durden, L. (Eds.), *Medical and Veterinary Entomology*. Boston: Academic Press, pp. 303-316.

KRISTJANSON, P. SWALLOW, B. ROWLANDS, G. KRUSKA, R. & DE LEEUW, P. 1999. Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. *Agricultural Systems*, 59: 79-98.

LATIF, A. NTANTISO, L. & DE BEER, C. 2019. African animal trypanosomosis (nagana) in northern KwaZulu-Natal, South Africa: Strategic treatment of cattle on a farm in endemic area. *Onderstepoort Journal of Veterinary Research*, 86(1): a1639.

LEAK, S.G.A. 1999. Tsetse Biology and Ecology. The role in the epidemiology and control of trypanosomosis. Wallingford and New York: CAB International.

LEDOKA, M. 2008. Molecular characterization of trypanosomes commonly found in cattle, wild animals and tsetse flies in KwaZulu-Natal, South Africa, 2005-2007. MSc Dissertation. University of Pretoria, South Africa.

LENT, H. & WYGODZINSKY, P. 1979. Revision of the Triatominae (Hemiptera, Reduviidae) and their significance as vectors of Chagas' disease. *Bulletin of the American Museum of Natural History*, 163: 123-520.

MACLENNAN, K.J.R. 1970. The epizootiology of trypanosomiasis in livestock in West Africa. In Mulligan, H.W. (Ed.), *The African Trypanosomiases*. London: Gearge Allen and Unwin, pp. 751-765.

MAHMOUD, M.M. & ELMALIK, K.H. 1977. Trypanosomiasis: goats as a possible reservoir of *Trypanosoma congolense* in the Republic of the Sudan. *Tropical Animal Health and Production*, 9: 167-170.

MAJIWA, P.A. MAINA, M. WAITUMBI, J.N. MIHOK, S. & ZWEYGARTH, E. 1993. *Trypanosoma* (*Nannomonas*) *congolense*: molecular characterization of a new genotype from Tsavo, Kenya. *Parasitology*, 106: 151-162.

MAJIWA, P. MASAKE, R. NANTULYA, V. HAMERS, R. & MATTHYSSENS, G. 1985. *Trypanosoma* (*Nannomonas*) *congolense*: identification of two karyotypic groups. *EMBO Journal*, 4: 3307-3313.

© University of Pretoria

MAMABOLO, M. NTANTISO, L. LATIF, A. & MAJIWA, P. 2009. Natural infection of cattle and tsetse flies in South Africa with two genotypic groups of *Trypanosoma congolense*. *Parasitology*, 136: 425-431.

MAMOUDOU, A. DELESPAUX, V. CHEPNDA, V. HACHIMOU, Z. ANDRIKAYE, J.P. ZOLI, A. & GEERTS, S. 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. *Acta Tropica*, 106: 115-118.

MARCOTTY, T. SIMUKOKO, H. BERKVENS, D. VERCRUYSSE, J. PRAET, N. & VAN DEN BOSSCHE, P. 2008. Evaluating the use of packed cell volume as an indicator of trypanosomal infections in cattle in eastern Zambia. *Preventive Veterinary Medicine*, 87: 288-300.

MASIGA, D.K. MCNAMARA, J.J. LAVEISSIÈRE, C. TRUC, P. & GIBSON, W.C. 1996. A high prevalence of mixed trypanosome infections in tsetse flies in Sinfra, Côte d'Ivoire, detected by DNA amplification. *Parasitology*, 112: 75-80.

MATOVU, E. MUGASA, C.M. WAISWA, P. KITIBWA, A. BOOBO, A. & NDUNG'U, J.M. 2020. Haemoparasitic infections in cattle from a *Trypanosoma brucei* rhodesiense sleeping sickness endemic district of Eastern Uganda. *Tropical Medicine and Infectious Disease*, 5(1): 24-33.

MATTHEWS, K. 2005. The developmental cell biology of *Trypanosoma brucei*. *Journal of Cell Science*, 118: 283-290.

MATTHEWS, K.R. MCCULLOCH, R. & MORRISON, L.J. 2015. The within-host dynamics of African trypanosome infections. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 370(1675): 20140288.

MATTIOLI, R.C. & FAYE, J.A. 1996. A comparative study of the parasitological buffy coat technique and an antigen enzyme immunoassay for trypanosome diagnosis in sequential *Trypanosoma congolense* infections in N'Dama, Gobrazebu and N'Dama × Gobrazebu cross-bred cattle. *Acta Tropica*, 62: 71-81.

© University of Pretoria

MCLAUGHLIN, G.L. SSENYONGA, S.S. NANTEZA, E. RUBAIRE-AKIKI, WAFULA, O. HANSEN, R.D. VODKIN, M.H. NOVAK, R.J. GORDON, V.R. MONTENEGRO-JAMES, S. JAMES, M. AVILES, H. ARMIJOS, R. SANTRICH, C. WEIGLE, K. SARAVIA, N. WOZNIAK, E. GAYE, O. MDACHI, R. SHAPIRO, S.Z. CHANG, K.P. & KAKOMA, I. 1996. PCR-based detection and typing of parasites. In Azcel, M.A. & Alkan, M.Z. (Eds.), *Parasitology for the 21st Century*. Oxfordshire: CAB International, pp. 261-287.

MIHOK, S. MARAMBA, O. MUNYOKI, E. & KAGOIYA, J. 1995. Mechanical transmission of *Trypanosoma spp*. by African Stomoxyinae (Diptera: Muscidae). *Tropical Medicine and Parasitology*, 46: 103-105.

MONCAYO, A. & SILVEIRA, A.C. 2007. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. *Memórias do Instituto Oswaldo Cruz*, 104(Suppl. I): 17-30.

MOSER, D.R. COOK, G.A. OCHS, D.E. BAILEY, C.P. MCKANE, M.R. & DONELSON, J.E. 1989. Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology*, 99: 57-66.

MOTLOANG, M. 2012. Vector competence of *Glossina austeni* and *G. brevipalpis* and characterization of *Trypanosoma congolense* strains from northern Kwazulu-Natal, South Africa. PhD Thesis. University of Pretoria, South Africa.

MOTLOANG, M.Y. MASUMU, J. MANS, B. VAN DEN BOSSCHE, P. & LATIF, A.A. 2012. Vector competence of *Glossina austeni* and *Glossina brevipalpis* for *Trypanosoma congolense* in KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, 79(1): 6-11.

MOTI, Y. FIKRU, R. BÜSCHER, P. VAN DEN ABBEELE, J. DUCHATEAU, L. & DELESPAUX, V. 2014. Detection of African animal trypanosomes: The haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer. *Veterinary Parasitology*, 203: 253-258.

MURRAY, M. & DEXTER, T.M. 1988. Anaemia in bovine African trypanosomiasis: a review. *Acta Tropica*, 45: 389-432.

MURRAY, M. & TRAIL, J.C.M. 1987. Comparative epidemiology and control of trypanosomes. *International Journal for Parasitology*, 17(2): 621-627.

© University of Pretoria

MUSINGUZI, S. SUGANUMA, K. ASADA, M. LAOHASINNARONG, D. SIVAKUMAR, T. YOKOYAMA, N. NAMANGALA, B. SUGIMOTO, C. SUZUKI, Y. XUAN, X. & INOUE, N. 2016. A PCR-based survey of animal African trypanosomosis and selected piroplasm parasites of cattle and goats in Zambia. *Journal of Veterinary Medical Science*, 78: 1819-1824.

N'DJETCHI, M.K. ILBOUDO, H. KOFFI, M. KABORÉ, J. KABORÉ, J.W. KABA, D. COURTIN, F. COULIBALY, B. FAURET, P. KOUAKOU, L. RAVEL, S. DEBORGGRAEVE, S. SOLANO, P. DE MEEÛS, T. BUCHETON, B. & JAMONNEAU, V. 2017. The study of trypanosome species circulating in domestic animals in two human African trypanosomiasis foci of Côte d'Ivoire identifies pigs and cattle as potential reservoirs of *Trypanosoma brucei gambiense*. *PLOS Neglected Tropical Diseases*, 11(10): e0005993.

NG'AYO, M.O. NJIRU, Z.K. KENYA, E.U. MULUVI, G.M. OSIR, E.O. & MASIGA, D.K. 2005. Detection of trypanosomes in small ruminants and pigs in western Kenya: important reservoirs in the epidemiology of sleeping sickness?. *Kinetoplastid Biology and Disease*, 4: 5-11.

NGOMTCHO, S.C.H. WEBER, J.S. NGO BUM, E. GBEM, T.T. KELM, S. & ACHUKWI, M.D. 2017. Molecular screening of tsetse flies and cattle reveal different *Trypanosoma* species including *T. grayi* and *T. theileri* in northern Cameroon. *Parasites Vectors*, 10(1): 631-646.

NJIRU, Z.K. CONSTANTINE, C.C. GUYA, S. CROWTHER, J. KIRAGU, J.M. THOMPSON, A. & DAVILLA, A.M.R. 2004a. The use of ITS 1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitology Research*, 95: 186-192.

NJIRU, Z.K. MAKUMI, J.N. OKOTH, S. NDUNGU, J.M. & GIBSON, W.C. 2004b. Identification of trypanosomes in *Glossina pallidipes* and *G. longipennis* in Kenya. *Infection, Genetics and Evolution*, 4: 29-35.

NTANTISO, L. 2012. Bovine trypanosome prevalence at game/livestock interface of Hluhluwe-Umfolozi game reserve in KwaZulu-Natal province, South Africa. MSc Dissertation. University of Pretoria, South Africa.

NTANTISO, L. DE BEER, C. MARCOTTY, T. & LATIF, A. 2014. Bovine trypanosomosis prevalence at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research*, 81(1): 1-8.

© University of Pretoria

NYIMBA, P.H. KOMBA, E.V. SUGIMOTO, C. & NAMANGALA, B. 2015. Prevalence and species distribution of caprine trypanosomosis in Sinazongwe and Kalomo districts of Zambia. *Veterinary Parasitology*, 210(3-4): 125-130.

ODONGO, S. DELESPAUX, V. NGOTHO, M. BEKKELE, S. & MAGEZ, S. 2016. Comparative evaluation of the nested ITS PCR against the 18S PCR-RFLP in a survey of bovine trypanosomiasis in Kwale County, Kenya. *Journal of Veterinary Diagnostic Investigation*, 28(5): 589-594.

OLADELE, O.I. & ADENEGAN, K.O. 1998. Implications of small ruminant farmer's socio-economic characteristics for extension services in South Western Nigeria. In OLOGHOBO, A.D. & IYAYI, E.A. (Eds.), *The Nigeria Livestock Industry in the 21st Century*. Lagos: Publication of Animal Science Association of Nigeria pp. 243-246.

ORTIZ, P.A. MAIA DA SILVA, F. CORTEZ, A.P. LIMA, L. CAMPANER, M. PRAL, E.M. ALFIERI, S.C. & TEIXEIRA, M.M.G. 2009. Genes of cathepsin L-like proteases in *Trypanosoma rangeli* isolates: Markers for diagnosis, genotyping and phylogenetic relationships. *Acta Tropica*, 112: 249-259.

OSAER, S. GOOSSENS, B. KORA, S. GAYE, M. DARBOE, L. 1999. Health and productivity of traditionally managed Djallonke sheep and West African dwarf goats under high and moderate trypanosomosis risk. *Veterinary Parasitology*, 82: 101-119.

OSANYO, A. & MAJIWA, P.W. 2006. Restriction fragment length polymorphism (RFLP) analysis of PCR products amplified from 18S ribosomal RNA gene of *Trypanosoma congolense*. *Kenya Journal of Science and Technology. Series B, Biological Sciences*, 13(1): 38-42.

PACHECO, T.D.A. MARCILI, A. COSTA, A.P.D. WITTER, R. MELO, A.L.T. BOAS, R.V. CHITARRA, C.S. DUTRA, V. NAKAZATO, L & PACHECO, R.C. 2018. Genetic diversity and molecular survey of *Trypanosoma* (*Megatrypanum*) *theileri* in cattle in Brazil's western Amazon region. *Revista Brasileira de Parasitologia Veterinária*, 27(4): 579-583.

PARIS, J. MURRAY, M. & MCODIMBA, F. 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica*, 39: 307-316.

© University of Pretoria

PEREIRA DE ALMEIDA, P.J. NDAO, M. GOOSSENS, B. & OSAER, S. 1998. PCR primer evaluation for the detection of trypanosome DNA in naturally infected goats. *Veterinary Parasitology*, 80(2): 111-116.

PERRY, B.D. RANDOLPH, T.F. MCDERMOTT, J.J. SONES, K.R. & THORNTON, P.K. 2002. Investing in animal health research to alleviate poverty. Nairobi, Kenya: International Livestock Research Institute.

PICOZZI, K. CARRINGTON, M. & WELBURN, S. 2008. A multiplex PCR that discriminates between *Trypanosoma brucei brucei* and zoonotic *T. b. rhodesiense*. *Experimental Parasitology*, 118(1): 41-46.

PIRON, M. FISA, R. CASAMITJANA, N. LÓPEZ-CHEJADE, P. PUIG, L. VERGÉS, M. GASCÓN, J. PRAT, J.G. PORTÚS, M. & SAULEDA, S. 2007. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta Tropica*, 103: 195-200.

RAYMOND, H.L. & ROUSSEAU, F. 1987. Abundance of horse flies (Diptera, Tabanidae) and reactions of cattle in a traditional farm of French Guiana. *Acta Oecologica*, 8: 125-134.

RASSI, A JR. RASSI, A. & MARCONDES DE REZENDE, J. 2012. American trypanosomiasis (Chagas disease). *Infectious Disease Clinics of North America*, 26: 275-291.

RODRIGUES, A.C. CAMPANER, M. TAKATA, C.S. DELL' PORTO, A. MILDER, R.V. TAKEDA, G.F. & TEIXEIRA, M.M.G. 2003. Brazilian isolates of *Trypanosoma* (*Megatrypanum*) *theileri*: diagnosis and differentiation of isolates from cattle and water buffalo based on biological characteristics and randomly amplified DNA sequences. *Veterinary Parasitology*, 116: 185-207.

RODRIGUES, A.C. GARCIA, H.A. ORTIZ, P.A. CORTEZ, A.P. MARTINKOVIC, F. PAIVA, F. BATISTA, J.S. MINERVINO, A.H. CAMPANER, M. PRAL, E.M. ALFIERI, S.C. & TEIXEIRA, M.M.G. 2010. Cysteine proteases of *Trypanosoma* (*Megatrypanum*) *theileri*: Cathepsin L-like gene sequences as targets for phylogenetic analysis, genotyping diagnosis. *Parasitology International*, 59: 318-325.

RODRIGUES, A. FIGHERA, R.A. SOUZA, T.M. SCHILD, A.L. SOARES, M.P. MILANO, J. & BARROS, C.S.L. 2005. Outbreaks of trypanosomiasis in horses by *Trypanosoma evansi* in the state of Rio Grande do Sul, Brazil: epidemiological, clinical, hematological, and pathological aspects. *Pesquisa Veterinária Brasileira*, 25: 239-249.

© University of Pretoria

SCHOFIELD, C.J. & MAUDLIN, I. 2001. Trypanosomiasis control. *International Journal for Parasitology*, 31: 614-619.

SECK, M.T. BOUYER, J. SALL, B. BENGALY, Z. & VREYSEN, M.J.B. 2010. The prevalence of African animal trypanosomoses and tsetse presence in Western Senegal. *Parasite*, 17: 257-265.

SEIFI, H.A. 1995. Clinical trypanosomosis due to *Trypanosoma theileri* in a cow in Iran. *Tropical Animal Health and Production*, 27: 93-94.

SHARMA, P. JUYAL, P. SINGLA, L. CHACHRA, D. & PAWAR, H. 2012. Comparative evaluation of real time PCR assay with conventional parasitological techniques for diagnosis of *Trypanosoma evansi* in cattle and buffaloes. *Veterinary Parasitology*, 190: 375-382.

SHAW, A. CECCHI, G. WINT, G. MATTIOLI, R. & ROBINSON, T. 2014. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. *Preventive Veterinary Medicine*, 113: 197-210.

SIGAUQUE, I. VAN DEN BOSSCHE, P. MOIANA, M. JAMAL, S. & NEVES, L. 2000 The distribution of tsetse (Diptera: Glossinidae) and bovine trypanosomosis in the Matutuine District, Maputo Province, Mozambique. *Onderstepoort Journal of Veterinary Research*, 67: 167-172.

SILBERMAYR, K. LI, F. SOUDRÉ, A. MÜLLER, S. & SÖLKNER, J. 2013. A novel qPCR assay for the detection of African animal trypanosomosis in trypanotolerant and trypanosusceptible cattle breeds. *PLOS Neglected Tropical Diseases*, 7(8): e2345.

SIMO, G. SILATSA, B. FLOBERT, N. LUTUMBA, P. MANSINSA, P. MADINGA, J. MANZAMBI, E. DE DEKEN, R. & ASONGANYI, T. 2012. Identification of different trypanosome species in the mid-guts of tsetse flies of the Malanga (Kimpese) sleeping sickness focus of the Democratic Republic of Congo. *Parasites & Vectors*, 5: 201.

SIMO, G. SOBGWI, P.F. NJITCHOUANG, G.R. NJIOKOU, F. KUIATE, J.R. CUNY, G. & ASONGANYI, T. 2013. Identification and genetic characterization of *Trypanosoma congolense* in domestic animals of Fontem in the South-West region of Cameroon. *Infection, Genetics and Evolution*, 18: 66-73.

© University of Pretoria

SIMUKOKO, H. MARCOTTY, T. PHIRI, I. GEYSEN, D. VERCRUYSSE, J. & VAN DEN BOSSCHE, P. 2007. The comparative role of cattle, goats and pigs in the epidemiology of livestock trypanosomiasis on the plateau of eastern Zambia. *Veterinary Parasitology*, 147: 231-238.

SIMWANGO, M. NGONYOKA, A. NNKO, H. SALEKWA, L. OLE-NESELLE, M. KIMERA, S. & GWAKISA, P. 2017. Molecular prevalence of trypanosome infections in cattle and tsetse flies in the Maasai Steppe, northern Tanzania. *Parasites & Vectors*, 10: 507-517.

SMITH, M.C. & SHERMAN, D.M. 1994. Blood, lymph, and immune system. In Smith, M.C. & Sherman, D.M. (Eds.), *Goat medicine*. 2nd edition. Philadelphia: Lea & Febiger, pp. 275-317.

STEPHEN, L.E. 1970. Clinical manifestations of the trypanosomiases in livestock and other domestic animals. In Mulligan, H.W. (Ed.), *The African trypanosomiases*. London: Gearge Allen and Unwin, pp. 774-794.

STEVENS, J.R. & BRISSE, S. 2004. Systematics of trypanosomes of medical and veterinary importance. In Maudlin, I. Holmes, P.H. & Miles, M.A. (Eds.), *The trypanosomiases*. Oxford: CAB International, pp. 1-23.

STEVENS, J.R. & GIBSON, W.C. 1999. The evolution of pathogenic trypanosomes. *Cadernos de Saúde Pública*, 15(4): 673-684.

STEVERDING, D. 2008. The history of African trypanosomiasis. *Parasites & Vectors*, 1: 3.

TAIOE, M. 2013. Molecular detection, genetic and phylogenetic analysis of trypanosome species in uMkhanyakude district of KwaZulu-Natal province, South Africa. MSc Dissertation. University of the Free State, South Africa.

TAMBUWAL, F.M. AGALE, B.M. & BANGANA, A. 2002. Haematological and serum biochemical values of apparently healthy red Sokoto goats. *Proceeding of 27th Annual Conference of Nigerian Society of Animal Production*, Akure, Nigeria, pp. 50-53.

TAYLOR, T. BOYLE, D. & BINGHAM, J. 2008. Development of a TaqMan PCR assay for the detection of *Trypanosoma evansi*, the agent of surra. *Veterinary Parasitology*, 153: 255-264.

© University of Pretoria

TCHAMDJA, E. KULO, A. VITOULEY, H. BATAWUI, K. BANKOLÉ, A. ADOMEFA, K. CECCHI, G. HOPPENHEIT, A. CLAUSEN, P. DE DEKEN, R. VAN DEN ABBEELE, J. MARCOTTY, T. & DELESPAUX, V. 2017. Cattle breeding, trypanosomosis prevalence and drug resistance in Northern Togo. *Veterinary Parasitology*, 236: 86-92.

TEKLE, T. TEREFE, G. CHERENET, T. ASHENAFI, H. AKODA, K.G. TEKO-AGBO, A. VAN DEN ABBEELE, J. GARI, G. CLAUSEN, P.H. HOPPENHEIT, A. MATTIOLI, R.C. PETER, R. MARCOTTY, T. CECCHI, G. DELESPAUX, V. 2018. Aberrant use and poor quality of trypanocides: a risk for drug resistance in south western Ethiopia. *BMC Veterinary Research*, 14(1): 4-11.

THRUSFIELD, M. 2005. Veterinary epidemiology. 3rd edition. Oxford, England: Blackwell Science.

TORR, S.J. & VALE, G.A. 2015. Know your foe: lessons from the analysis of tsetse fly behaviour. *Trends in Parasitology*, 31(3): 95-99.

TRAIL, J.C.M. D'IETEREN, G.D.M. COLARDELLE, C. MAILLE, J.C. ORDNER, G. SAUVEROCHE, B. & YANGARI, G. 1991. Evaluation of a field test for trypanotolerance in young N'Dama cattle. *Acta Tropica*, 48: 47-57.

UILENBERG, G. 1998. A field guide for the diagnosis, treatment and prevention of African animal trypanosomosis. Rome, Italy: Food and agriculture organization of the united nation.

URQUHART, G.M. ARMOUR, J. DUNCAN, J.L. DUNN, A.M. & JENNINGS, F.W. 1996. Veterinary parasitology. 2nd edition. Oxford, United Kingdome: Blackwell Science.

VALE, G.A. 1993. Development of baits for tsetse flies (Diptera: Glossinidae) in Zimbabwe. *Journal of Medical Entomology*, 30: 831-842.

VAN DEN BOSSCHE, P. 2000. The development of a new strategy for the sustainable control of bovine trypanosomosis in Southern Africa. PhD Thesis. University of Pretoria, South Africa.

VAN DEN BOSSCHE, P. ESTERHUIZEN, J. NKUNA, R. MATJILA, T. PENZHORN, B. GEERTS, S. & MARCOTTY, T. 2006. An update of the bovine trypanosomosis situation at the edge of Hluhluwe-Imfolozi Park, Kwazulu-Natal Province, South Africa. *Onderstepoort Journal of Veterinary Research*, 73: 77-79.

© University of Pretoria

VAN DEN BOSSCHE, P. & ROWLANDS, G. 2001. The relationship between the parasitological prevalence of trypanosomal infections in cattle and herd average packed cell volume. *Acta Tropica*, 78: 163-170.

VICKERMAN, K. TETLEY, L. HENDRY, K.A.K. & TURNER, C.M.R. 1988. Biology of African trypanosomes in the tsetse fly. *Biology of the cell*, 64: 109-119.

VREYSEN, M.J.B. SALEH, K.M. ALI, M.Y. ABDULLA, A.M. ZHU, Z.R. JUMA, K.G. DYCK, V.A. MSANGI, A.R. MKONYI, P.A. & FELDMANN, H.U. 2000. *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology*, 93(1): 123-135.

VREYSEN, M. SECK, M. SALL, B. & BOUYER, J. 2013. Tsetse flies: Their biology and control using area-wide integrated pest management approaches. *Journal of Invertebrate Pathology*, 112: S15-S25.

WALSHE, D.P. OOI, C.P. LEHANE, M.J. & HAINES, L.R. 2009. The enemy within: interactions between tsetse, trypanosomes and symbionts. *Advances in Insect Physiology*, 37:119-175.

WOLK, D. MITCHELL, S. & PATEL, R. 2001. Principles of molecular microbiology testing methods. *Infectious Disease Clinics of North America*, 15: 1157-1204.

XAVIER, S.C.C. ROQUE, A.L.R. BILAC, D. ARAÚJO, V.A.L. NETO, S.F.C. LOROSA, E.S. SILVA, L.F.C.F & JANSEN, A.M. 2014. Distantiae transmission of *Trypanosoma cruzi*: A new epidemiological feature of acute Chagas disease in Brazil. *PLOS Neglected Tropical Diseases*, 8(5): e2878.

YARO, M. MUNYARD, K.A. STEAR, M.J. & GROTH, D.M. 2016. Combatting African animal trypanosomiasis (AAT) in livestock: the potential role of trypanotolerance. *Veterinary Parasitology*, 225: 43-52.

© University of Pretoria

APPENDICES

Appendix 1.1: Research Ethics Committee approval from the University of Pretoria (REC098-18).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	Trypanosomosis in KwaZulu-Natal: The incidence of infection in cattle and the role of goats as reservoirs.
PROJECT NUMBER	REC098-18
RESEARCHER/PRINCIPAL INVESTIGATOR	Samantha Mnkandla

DISSERTATION/THESIS SUBMITTED FOR	MSc

SUPERVISOR	Luis Neves

APPROVED	Date 06 May 2019
CHAIRMAN: UP Research Ethics Committee	Signature

© University of Pretoria

Appendix 1.2: Animal Ethics Committee approval from the University of Pretoria (V095-18).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	*Trypanosomosis* in KwaZulu-Natal: The incidence of infection in cattle and the role of goats as reservoirs
PROJECT NUMBER	V095-18
RESEARCHER/PRINCIPAL INVESTIGATOR	S Mnkandla

STUDENT NUMBER (where applicable)	U_18392262
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES/SAMPLES	Cattle	Goats
NUMBER OF ANIMALS	60	384
Approval period to use animals for research/testing purposes		November 2018 – November 2019
SUPERVISOR	Prof. L Neves	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	26 November 2018
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

© University of Pretoria

Appendix 1.3: DAFF Section 20 approval.

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001
Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za

Dr S Mbizeni
ARC-Onderstepoort Veterinary Research
100 Old Soutpan Road
Onderstepoort, 0110

Email: MbizeniS@arc.agric.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Dr Mbizeni

Your application, submitted 25 October 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

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

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study may not commence until a valid ethics approval has been obtained, in writing, from the relevant ethics approval body;
4. Blood samples may only be collected from the dip tanks as stipulated in the Section 20 application submitted;
5. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);

© University of Pretoria

6. Only a registered waste disposal company may be utilised for the removal of waste generated during the study.

Title of research/study: "Assessing Animal Trypanosomosis prevalence in tsetse and livestock in north eastern KwaZulu-Natal"
Researcher: Dr S Mbizeni
Institution: ARC-Onderstepoort Veterinary Research
Your reference: AEC 31.18
Reference: 12/11/1/1
Expiry date: 30 November 2019

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -11- 15

SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

© University of Pretoria

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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

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

Dr S Mbizeni
ARC-Onderstepoort Veterinary Research
100 Old Soutpan Road
Onderstepoort, 0110

Email: MbizeniS@arc.agric.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ASSESSING ANIMAL TRYPANOSOMOSIS PREVALENCE IN TSETSE AND LIVESTOCK IN NORTH EASTERN KWAZULU-NATAL"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) All positive DNA extractions may be stored in liquid nitrogen at -20 to -80°C at the Epidemiology, Parasites and Vectors Section, ARC-OVR;

ii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -11- 15

© University of Pretoria