MOLECULAR CHARACTERIZATION OF TRYPANOSOMES COMMONLY FOUND IN CATTLE, WILD ANIMALS AND TSETSE FLIES IN KWAZULU-NATAL, SOUTH AFRICA, 2005-2007

BY

MPHO VICTORIA LEDOKA

Submitted in partial fulfilment of the requirements for the degree Magister Scientiae in the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria

Prof PA Majiwa
Prof AA Latif

November 2008

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Declaration

I (Mpho Victoria Ledoka) declare that the thesis which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

Signature ..... Date: 11 November 2008

Acknowledgement

The research reported here emanates from a project carried out at the Onderstepoort Veterinary Institute of the Agricultural Research Council in the Molecular Epidemiology and Diagnostics Programme. The research was carried out as part of the Professional Development Programme funded by the Department of Science and Technology, and the programme was administered by Ms Molope.

The successful conclusion of this project would not have been possible without: The expertise, guidance and support of my supervisor Prof Phelix Majiwa; Prof Abdalla Latif, my co-supervisor, for assistance with all the sampling; Dr Erich Zweygarth, for help with the culture work; Dr Lundi Ntantiso, State Veterinarian of KZN; Dr Peter Van den Bossche for donating some trypanosome isolates; Staff at Biotechnology building, ARC-OVI for technical and moral support ; Staff at Parasitology building for help with the laboratory animals; Staff at Entomology for looking after the tsetse flies; KZN Provincial Department of Agricultural and Environmental Affairs; Wildlife Conservation groups working in KZN; Game Park services and Fellow “DST students”.

To my parents, Mr and Mrs Ledoka, thanks for your unending support throughout my studies. I also want to acknowledge the moral support, motivation and love of my husband Mankabidi Cuthbert Mamabolo.

Last but not least I give all honour to God almighty for the strength to begin and complete this project.

Ecclesiastes 7:8

Better is the end of a thing than the beginning thereof…

List of Contents

List of Figures

List of Tables

Acronyms and Abbreviations

ARC	Agricultural Research Council
BHC	Benzene hexachloride
DDT	Dichloro-Diphenyl-Trichloroethane
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide-triphosphate
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-Linked Immuno-Sorbent Assay
HCH	Hexachlorocyclohexane
KZN	KwaZulu Natal
MEM	Minimum Essential Medium
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase Chain Reaction
PSG	2% glucose in phosphate buffered saline
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RG	RAPD group
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic Acid
SIT	Sterile Insect Technique
T_a	Annealing temperature
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
UP	University of Pretoria
UV	Ultraviolet
VAT	Variable Antigen Types
VSG	Variant Surface Glycoprotein

Summary

MOLECULAR CHARACTERIZATION OF TRYPANOSOMES COMMONLY FOUND IN CATTLE, WILD ANIMALS AND TSETSE FLIES IN KWAZULU-NATAL, SOUTH AFRICA, 2005-2007

By

MPHO VICTORIA LEDOKA

Promoter: Prof PA Majiwa
Co-promoter: Prof AA Latif
Department: Veterinary Tropical Diseases
Degree: MSc

The objective of this study was to use molecular biological reagents (primers targeting different genomic loci) and methods (PCR and RFLP) to detect and characterize trypanosomes in cattle, wild animals and tsetse flies in KwaZulu-Natal, thus contributing to improved understanding of the genetic diversity of trypanosome species infecting cattle at the game/livestock interface in the KwaZulu-Natal Province of South Africa.
Primers based on sequences of different loci in the trypanosome genome were used in conducting polymerase chain reactions (PCRs) on samples collected from cattle at 14 diptanks and one commercial farm around the Hluhluwe-Umfolozi Game Reserve, wild animals within Hluhluwe-Umfolozi Game Reserve and tsetse flies from the commercial farm, Hluhluwe-Umfolozi Game Reserve and two other Game Reserves. Trypanosome isolates were grown under laboratory conditions in cattle, rodents and culture medium for molecular characterizations. Overall, a total of 673 cattle, 266 tsetse flies, 141 buffaloes and 6 rhinoceros samples were analyzed.

The following observations were made: two species of trypanosomes are present in KZN; *T. congolense* and *T. vivax.* The two species were found as single and as mixed infections in cattle.

There are two genotypic groups of *T. congolense* in KZN; the Savannah- and the Kilifi-type. The two genotypic groups were found as mixed infections in cattle and in tsetse flies. Lastly, there are at least five “genomic variants” of Savannah-type *T. congolense* in KZN.

The infection rate in cattle ranged from 5.2-91%; in tsetse flies, 11-97.5% and in wild animals it was 4.3%.

Mixed infection of *T. congolense* and *T. vivax* were only observed in samples from one diptank. Mixed infections of Savannah- and Kilifi-type *T. congolense* were observed in samples from Boomerang commercial farm, and in tsetse flies.

CHAPTER 1

1. INTRODUCTION

The history of trypanosomosis in South Africa, particularly in what was then known as Zululand, which is the low-lying north-eastern part of the now KwaZulu-Natal, from the Tugela River to the Mozambique border, dates back to the nineteenth century. The disease was known there as *nagana*, which means to be depressed or low in spirit, describing the state of an animal in the chronic stages of infection with trypanosomes. In 1895 Bruce discovered that trypanosomes were the organisms causing *nagana* (Bruce, 1895). He also discovered that wild animals served as reservoirs for the parasites, which are mostly transmitted by tsetse flies. *Nagana* represents a serious problem in most of Africa, specifically in sub-Saharan Africa (Connor and Van den Bossche, 2004).

The occurrence of trypanosomosis in Africa largely depends on the presence of tsetse flies. They infest 36 countries with a combined total area of between 9 and 10 million km^2, where close to 50 million cattle and tens of millions of small ruminants are at risk of being infected with trypanosomes. Trypanosomosis is estimated to cost sub-Saharan Africa approximately US $4 billion each year, calculated as direct losses in meat production, milk yield, loss in traction and transport and programs that control the disease. By denying agricultural communities the use of animals for traction, only small areas of land can be tilled by hand, leaving the affected communities vulnerable to food shortages, starvation and famine (FAO, 2003).

The first record of the presence of tsetse flies in South Africa was in 1836. To date, the species of tsetse flies recorded are *Glossina morsitans morsitans, G. pallidipes, G. austeni* Newstead and *G. brevipalpis* Newstead. In 1897 *G. m. morsitans* completely disappeared from South Africa after the number of cattle and antelopes and other wild bovidae was reduced following the rinderpest epizootic of 1896-1897. The predominant tsetse flies in the late 19th century were *G. pallidipes* which were regarded as the most common vectors of pathogenic trypanosomes. This fly species was eradicated in 1954 by aerial spraying (Du Toit, 1954). The other tsetse flies were *G. austeni* Newstead and *G. brevipalpis* Newstead. The tsetse-transmitted trypanosomes that were commonly found in the area were:

Trypanosoma brucei, *T. congolense* and *T. vivax* (Kappmeier *et al.,* 1998*)*. Presently trypanosomes are transmitted by at least two tsetse species: *G. austeni* Newstead and *G. brevipalpis* Newstead (Esterhuizen *et al.,* 2006). Apart from the survey conducted by Van den Bossche *et al.* (2006b), no accurate information exists regarding the current incidence of trypanosomosis in South Africa, and the data collected so far has been obtained using microscopy on blood smears. Application of more sensitive methods could clarify the issue.

KwaZulu-Natal is the only province in South Africa with cases of *nagana.* Here the disease is confined to the magisterial districts of Ingwavuma, Ubombo, Hlabisa and Nongoma. The combined tsetse-infested area in the four districts covers 16 000km^2, and is inhabited by approximately 426 000 humans. Close to 10 000 of the 360 000 cattle in these districts belong to commercial farmers, and the rest are owned by developing farmers in communal farming areas (Kappmeier and Nevill, 1999). Hluhluwe-Umfolozi and Mkuze are two of the largest game reserves in the districts.

By gathering accurate data regarding the epidemiology of trypanosomosis in the areas within and outlaying the game reserves, development of control strategies that can protect livestock against trypanosomosis can be implemented in the management of game parks and in the communities that live nearby the game parks. The research documented here contributes to improved understanding of the trypanosome species infecting cattle at the game-livestock interface in KZN.

Molecular tools were used to characterize trypanosomes in cattle, wild animals and tsetse flies in KZN. In so doing, it has been possible to determine the different species of trypanosomes in cattle, wild animals and tsetse flies, the prevalent species of trypanosomes, and to investigate whether different genotypic groups of *T. congolense* are present in KZN. The genetic diversity of Savannah-type *T. congolense* isolates from KZN was also investigated.

CHAPTER 2

2. LITERATURE REVIEW

2.1 General introduction

Trypanosomes are flagellated protozoa that belong to the genus *Trypanosoma.* They cause diseases that are generally referred to as trypanosomosis. These protozoa can infect all classes of vertebrates: fish, amphibians, reptiles, birds and mammals (Connor and Van den Bossche, 2004). Hence, the clinical manifestation of the disease depends on the host and the infecting species. For example, *Trypanosoma cruzi* causes chagas disease in humans; *T. evansi* causes surra in camels and horses; *T. brucei rhodesiense* and *T. brucei gambiense* cause sleeping sickness in humans; and, of importance in this study, *T. congolense, T. vivax* and *T. brucei brucei* cause *nagana* in livestock.

Evans was the first person to associate trypanosomes with disease. This was after he observed the parasites in the blood of camels and horses that had a disease that was known in India as *surra*, now known to be caused by *T. evansi* (Evans, 1880).

2.2 Trypanosomes

Trypanosomes belong to the phylum of *Sarcomastigophora*, class *Zoomastogophorea*, the order of *Kinetoplastidae*, the family of *Trypanosomatidae*, and the genus of *Trypanosoma*. Trypanosomes that infect mammals have been divided into two groups, Stercoraria and Salivaria. The former is further divided into three subgenera: *Megatrypanum, Schizotrypanum* and *Herpetosoma*. With the exception of a few human cases, trypanosomes that belong to the *Herpetosoma* subgenus (*T. lewisi, T. musculi* and *T. microtis*) parasitize rodents. *Trypanosoma theileri* belong to the *Megatrypanum* subgenus, whereas *T. cruzi* and *T. rangeli* belong to *Schizotrypanum* subgenus.

Salivarian trypanosomes are divided into four subgenera: the subgenus *Pycnomonas, Nannomonas, Duttonella* and *Trypanozoon.* The subgenus *Pycnomonas* contains only one species, *T. suis.* This species has received limited attention, perhaps because it is of little economic importance. The subgenus *Nannomonas* contains two recognized species; *T. congolense* and *T. simiae* and a proposed new one, *T. godfreyi* (McNamara *et al.,* 1994). All three species are morphologically identical but differ with respect to host specificity and disease symptoms in the host. Furthermore, *T. congolense* is made up of four genotypic

groups: the Savannah, Kilifi, Tsavo and West African Riverine Forest. The subgenus *Trypanozoon* contains three species: *T. equiperdum, T. evansi* and *T. brucei.* The latter species is the only member of this subgenus that can undergo cyclic transmission by tsetse flies. Mechanical transmission of *T. equiperdum* and *T. evansi* by the tsetse vector is however possible. *Trypanosoma brucei* is further divided into three subspecies; *T. brucei gambiense, T. b. rhodesiense* and *T. b. brucei.* *T. vivax* is in the subgenus *Duttonella* (Stevens and Brisse, 2004).

2.2.1 Morphology

Trypanosomes are whip-like unicellular organisms, of 8-50µm in size, depending on the species. In Figure 2.1 is a simplified sketch of a trypanosome (trypomastigote) (Uilenberg, 1998). *Trypanosoma congolense,* the smallest of the three livestock-infective trypanosomes, is between 8 and 20µm in length, *T. vivax* is between 20 and 26µm and *T. brucei* is between 23 and 30µm. A number of cellular organelles can be clearly seen under an electron microscope. In the trypomastigote, the kinetoplast is situated near the posterior end, the flagellum can be seen arising from the parabasal body, the undulating membrane is seen along the length of the body and the nucleus is placed in the centre. The kinetoplast is distinct and well-defined, and its size and position differs among species. It plays an important role in reproduction, metabolism and in the cyclic transmission of trypanosomes. The flagellum is used for movement through the blood plasma and tissue fluid (Uilenberg, 1998).

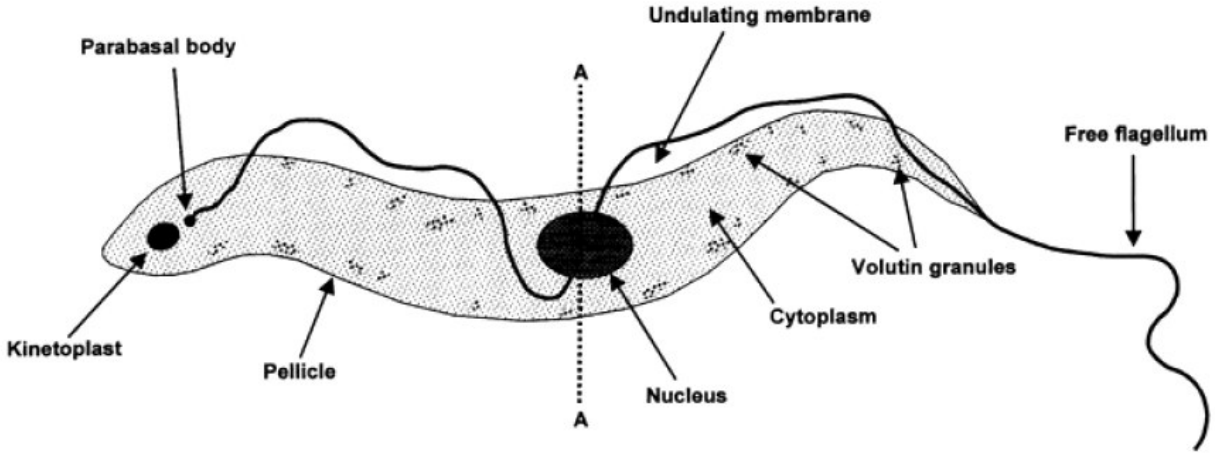

Figure 2.1: A simplified drawing of a trypanosome (Taken from Uilenberg, 1998).

Trypanosomes can spend a long time in the blood of their mammalian host, where they are exposed to constant immune attack. To escape the immune response of their hosts, they

periodically change their surface glycoprotein coat, and this takes place at a frequency of between 10^{-2} and 10^{-7} per cell generation (Vanhamme *et al*., 2001). The parasite's surface glycoprotein coat is changed in the course of antigenic variation, hence called the "variant surface glycoprotein" (VSG). The VSG is abundant and immunogenic, composed of 500 amino acids and can be between 55kDa and 65kDa in size depending on the trypanosome species. The function of the VSG is to form an immuno-protective coat that shields invariant antigens of the parasite against innate and acquired immune mechanisms of their hosts (Vanhamme *et al*., 2001; Morrison *et al*., 2005).

2.2.2 Reproduction

Trypanosomes reproduce asexually by binary fission, i.e., the division of one parent cell into two daughter cells. They can also reproduce sexually whereby genetic exchange takes place between two of them. This process is known to take place in the tsetse vectors but the frequency of its occurrence is unknown (Jenni *et al*., 1986; Uilenberg, 1998) and the process is not obligatory.

2.2.3 Life cycle

Typically trypanosomes require two hosts to complete their lifecycle which is made up of different developmental forms (Figure 2.2). The stumpy bloodstream forms of *T. brucei* are ingested by tsetse flies when they feed on the blood of an infected animal. In the midgut, the bloodstream forms transform into procyclic trypanomastigotes that penetrate the peritrophic membrane and eventually enter the proventriculus where they transform into mesocyclic trypanomastigotes. These migrate to the salivary glands where the mesocyclis transform into epimastogotes. The latter forms then multiply and differentiate into metacyclic forms which are ready to be transmitted by a tsetse fly when it feeds on a mammalian host (Vickerman *et al.,* 1988; Hendricks *et al*., 2000). The developmental stages of *T. congolense* in a tsetse fly are similar to those of *T. brucei* except that the epimastigotes multiply and differentiate into metacyclic trypanomastigotes in the proboscis. Unlike the two mentioned species, none of the developmental stages of *T. vivax* occur in the midgut, instead, the stumpy bloodstream forms transform into procyclic trypanomastigotes, then into epimastigotes in the foregut. These then migrate to the proboscis where they differentiate into metacyclic trypanomastigotes (Vickerman *et al.,* 1988).

During a blood meal metacyclics that matured in either the proboscis or salivary glands are transferred with the saliva into the dermis of the skin. In the mammalian host the metacyclics differentiate into different bloodstream forms, i.e., the slender, intermediate and then stumpy forms one and two. At this stage the life cycle of a trypanosome would be complete, and the stumpy forms can be ingested by a tsetse fly and undergo cyclical changes again (Hendricks *et al.*, 2000).

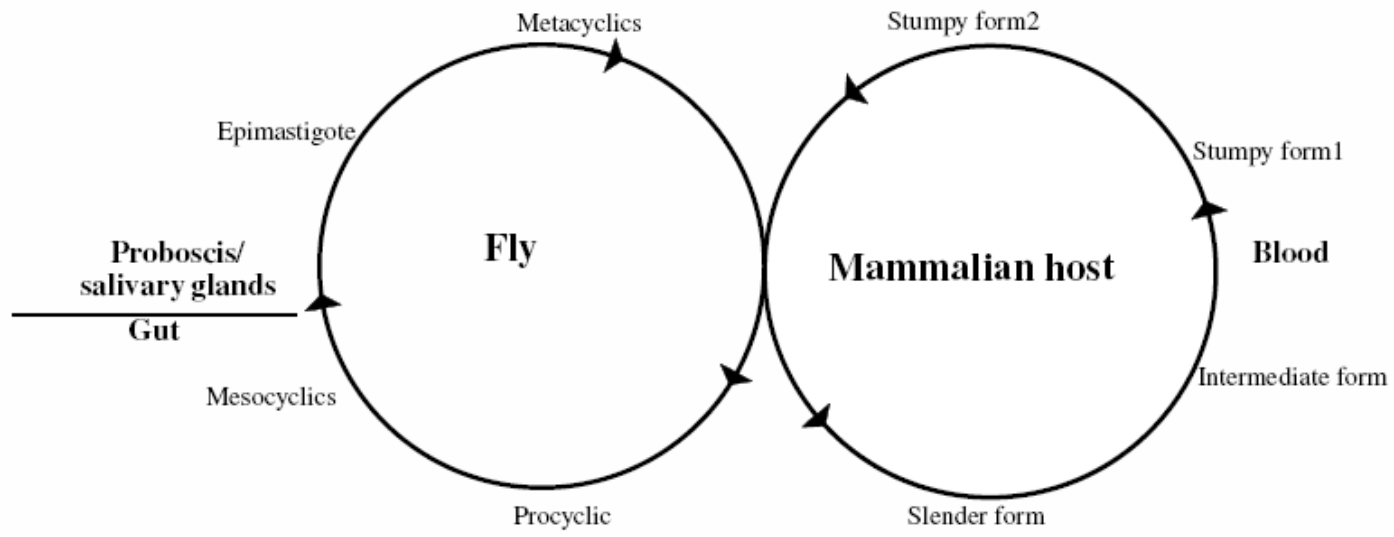

Figure 2.2: Generalized diagrammatic illustration of the life cycle of salivarian trypanosomes (Adapted from Hendricks *et al.*, 2000).

2.2.4 Transmission

Trypanosomes are primarily transmitted from host to host by insect vectors, cyclically or mechanically. Cyclic transmission refers to the developmental cycle of the parasite in the tsetse fly vector (as detailed in section 2.2.3). Biting flies such as *Tabanus, Stomoxys* and *Lyperosia* also transmit trypanosomes, but they do so mechanically. Other methods of transmission of trypanosomes are venereal, experimental and vertical. Venereally transmitted parasites like *T. equiperdum* are transferred during coitus. Experimentally, trypanosomes may be transmitted by the use of needles and syringes containing trypanosomes. Vertical transmission (from mother to offspring) is also possible whereby the parasites can cross the placental membrane and infect the foetus (Katakura *et al.,* 1997; Sekoni, 1994).

2.3 Tsetse flies

Tsetse flies belong to the phylum Athropoda, class Insecta, the order Diptera, family Glossinidae, and genus *Glossina*. They are of economic importance because they transmit different species of trypanosomes.

A total of 31 species and subspecies belonging to this genus have been grouped into three subgenera (*fusca, morsitans* and *palpalis*) based on habitat preference and the structure of the genitalia. The *fusca* group is mostly found in the rain forest or heavy riparian forest. The genitalia of the females have an external armature with five plates. Most species in this subgenus have a well developed signum (Leak, 1998). The *morsitans* group is mainly restricted to the savannah woodlands and the genitalia of the females have an external armature with three plates. Males in this subgenus are identified by the morphology of the superior claspers. The *palpalis* group also inhabits the rain forest, and some of the species extend into the savannah. The genitalia of the females have an external armature with six plates. Males are identified by their inferior claspers (Phelps and Lovemore, 1994; Leak, 1998).

In addition to having preferred habitats, tsetse flies have preferred hosts upon which they feed. In the absence thereof, they feed on other species. They feed exclusively on blood, and they locate their hosts by vision (movement and colour) and odour (volatile substances in the urine and breath). If they happen to feed on an infected animal, they become infected after which they act as vectors, transmitting the infection to animals on which they subsequently feed. Tsetse flies are efficient vectors of trypanosomes because once they are infected they remain so for the rest of their lives (Gibson and Bailey, 2003).

2.4 Common reservoirs of trypanosomes

More than 30 species of wild animals can be infected with pathogenic trypanosomes but remain carriers or reservoirs of the organisms because they do not show manifestation of the disease. This attribute is believed to be due to the fact that trypanosomes have been in contact with wild animals for a long time and they have become adapted to each other. The following animals have been found to harbour pathogenic trypanosomes of man and animals: buffalo, bushbucks, chimpanzee, eland, elephant, hartebeest, hippopotamus, hyena, kudu, lion, monkey, impala, and zebra, to mention a few (Connor and Van den Bossche, 2004).

2.5 Trypanosomosis

2.5.1 Signs and symptoms in cattle

Generally clinical signs and symptoms of *nagana* in cattle include intermittent fever, oedema, emaciation, pale mucous membranes due to anaemia, enlarged lymph nodes, rough and dull hair coats, occasional diarrhoea and infertility. Infected cows usually abort, and the ejaculates of infected bulls have reduced live spermatozoa with reduced motility as a result of testicular damage (Sekoni *et al.,* 1988). Figure 2.3 shows a typical emaciated animal found to be infected with trypanosomes. In endemic areas clinical signs alone can be non-specific for diagnosis of *nagana* because other disease states can result in similar symptoms. The morbidity and mortality of infected animals largely depend on the breed and age of the animal, and virulence and dose of the infecting organism (OIE, 2003).

2.5.2 Diagnosis of trypanosomosis

Animals in trypanosomosis endemic areas should be suspected of infection when they are in a poor condition and present typical clinical signs of *nagana*. To confirm infection, parasites must either be seen in the blood, buffy coat or lymph node smear of the animal by microscopy, antibodies to the parasite or antigens of the parasite must be detected in the blood of the animal by antibody or antigen ELISA and/or trypanosome DNA must be amplified by PCR. The disadvantage of microscopy is that it is labour intensive and the sensitivity of the technique is lower than the recently developed tests (Cox *et al.,* 2005). The specificity of the monoclonal antibody-based ELISA for detection of trypanosome antigens was shown to be good, although the sensitivity was unsatisfactory. In addition, one ELISA test cannot differentiate species of trypanosomes (Eisler *et al*., 1998). Antibody detection ELISA was a

great improvement on sensitivity, but it can give false negatives when animals are in the chronic state of the disease (Rebeski *et al.,* 2000).

Figure 2.3: A typical emaciated animal (to the right, with pointed arrow) was found to be infected with trypanosomes. The photograph was taken at Mahlambinyathi diptank, KwaZulu-Natal, November 2005. Photograph courtesy of Prof Majiwa.

2.6 Characterization of trypanosomes

Initially, trypanosome species were characterized by morphology using stained blood smears (as in Figure 2.4), movement characteristics in fresh blood smears, tsetse transmissibility, and location of the parasites in specific organs of tsetse flies during cyclical development.

It is difficult to identify a parasite solely on the basis of its morphology. For instance, it is not possible to distinguish *T. simiae* from *T. congolense* or *T. brucei* from *T. evansi* because they are morphologically identical. Location of trypanosomes in the proboscis, gut or salivary gland of tsetse flies can to some extent be used for classification of these parasites. The presence of trypanosomes in the midgut and proboscis of the tsetse indicates infection with *Nannomonas* trypanosomes, the midgut and salivary glands indicates infection with *T. brucei* and in only the salivary glands indicates infection with *T. vivax.* The limitation of this method is that mixed infections of *Nannomonas* and *Duttonella* cannot be detected, and species that have identical developmental cycles in the vector cannot be differentiated. Another approach that has been used is to inoculate the parasite into experimental animals and observe its infectivity and virulence to the host. The disadvantage of this method is that not all species of trypanosomes grow in laboratory animals (Godfrey, 1961).

Molecular methods for characterization of trypanosomes include isoenzyme typing, orthogonal field alteration gel electrophoresis (OFAGE), DNA hybridization and PCR. By comparing the electrophoretic mobility of 12 enzymes in 78 stocks of *T. congolense,* two *Nannomonas* trypanosomes (Savannah and West African Riverine Forest) were differentiated for the first time (Young and Godfrey, 1983). OFAGE is a technique whereby chromosomes of sizes between 100kb and 1Mb are resolved. By analyzing the resulting chromosome profiles, it is possible to distinguish trypanosomes belonging to different serodemes (Majiwa *et al.,* 1986; Masake *et al.* 1988). The work carried out using isoenzyme typing and OFAGE was simplified by the development and use of DNA probes and later PCR. DNA probes are hybridized with a denatured DNA sample which is fixed on a filter matrix. The probe will then bind onto the complementary sequence of the denatured DNA if present. This technique was a great improvement in the accurate identification of trypanosomes, but it is time-consuming, laborious and of limited sensitivity (Masiga *et al.,* 1992). The use of PCR with appropriate primers overcomes the limitations presented by other methods of trypanosome characterization.

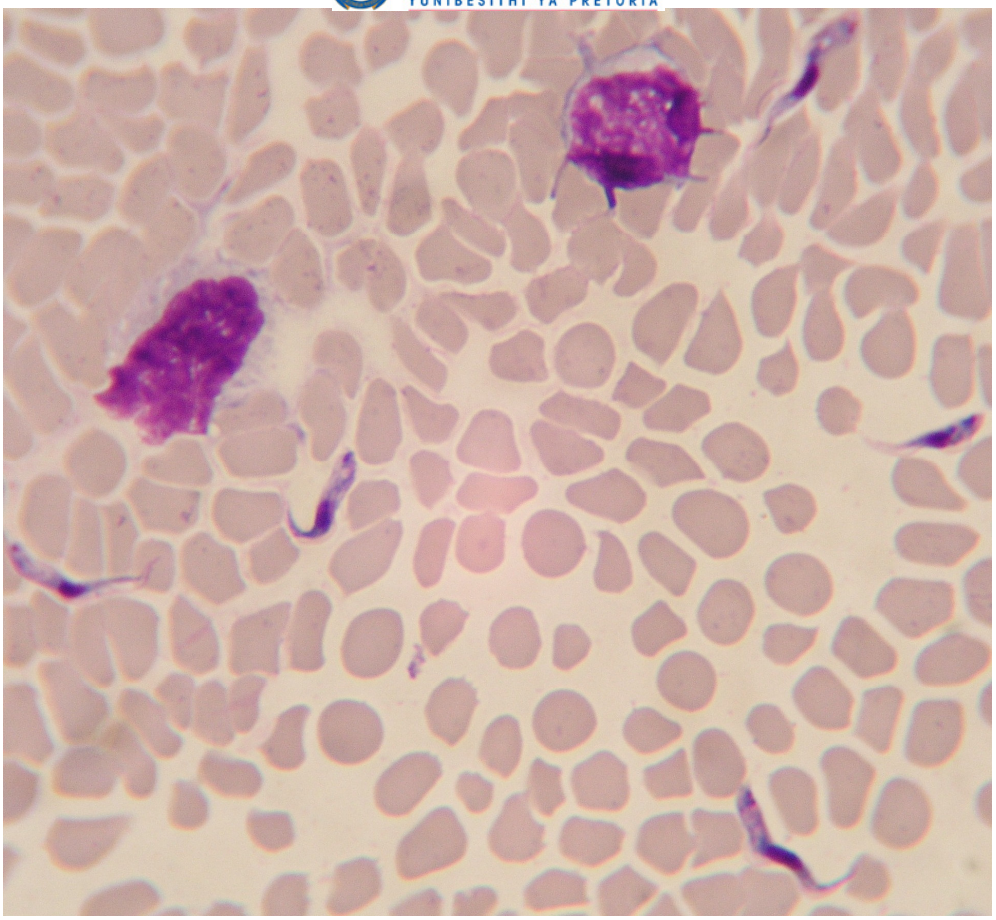

Figure 2.4: Trypanosomes in a Giemsa stained thin blood smear. Photograph courtesy of Prof Latif.

Highly repetitive DNA sequences (satellite DNA) make up a large proportion of the genome of trypanosomes. In *T. brucei,* they make up 12% of the genome; and in *T. cruzi,* 9% (Lanar *et al.,* 1981; Gonzalez *et al.,* 1984; Masiga *et al.,* 1996). Apart from the fact that they make up a bulk of minichromosomes, the exact percentage of repetitive DNA in the genomes of *Trypanozoon* and *Nannomonas* trypanosomes is not recorded. Nevertheless, these satellite DNAs were common targets in the development of species-specific DNA probes and later species-specific primers (Gibson *et al*., 1988). For *T. brucei* species, the satellite DNA of interest is a repetitive unit of 177bp, whereas for Savannah-type and Kilifi-type *T. congolense,* the repetitive units are 369bp and 368bp respectively (Sloof *et al.,* 1983b, Gibson *et al.,* 1987, Masiga *et al.,* 1992). Using repetitive DNA sequences as DNA probes or as targets for amplification in PCR greatly improved the accuracy of identification of trypanosomes because the sequences are unique to a particular subgenus, species or subspecies and they are present in high copies (Gonzalez *et al.,* 1984).

In contrast to the non-coding satellite DNA repeats of trypanosomes of the subgenus *Trypanozoon* and *Nannomonas,* the tandem repeat, which is also a target for PCR in the genome of trypanosomes of subgenus *Duttonella,* encodes a diagnostic antigen. The monomeric unit is 935bp and it appears to be conserved in the genome of *T. vivax* isolates from different origins (Masake *et al.,* 1997).

The limitation of using species-specific primers is that a pair of primers is required to detect each species of trypanosome or genotypic group of *T. congolense*. So, in order to accurately identify different species of trypanosomes, three to five different PCRs must be carried out per sample. This is impractical when a large number of samples has to be processed. In addition, this method of amplification only permits identification of trypanosomes for which there are species-specific primers. Desquesnes (Unpublished) attempted multiplex PCRs, thereby reducing the number of tests that need to be carried out with individual species-specific primers, but this resulted in reduced sensitivity and in the synthesis of non-specific products. The use of multiple primers to detect different species of trypanosomes necessitated the search for a universal and sensitive PCR that can detect trypanosomes in field samples, combined with species-specific identification. PCR amplifying the18S rRNA gene, combined with restriction enzyme length polymorphism (RFLP) analysis provided such a tool. The 18S PCR makes use of a single assay, instead of the various species-specific PCRs. The amplified region is present as a multi-copy locus (over 150 copies of the rDNA locus) and

encompasses a polymorphic area which is revealed when the PCR product is digested with *Eco*571 and *Msp*I. The resulting RFLP profiles are distinct for the different species of trypanosomes. In addition, there is no homology with DNA sequences of non-trypanosomatid parasites that infect cattle (Geysen *et al.,* 2003). When used in PCR, primers that target this locus detect only trypanosomatids. Amplification of the segment of the 18S rRNA gene is thought to be higher in sensitivity compared to the 'pan-trypanosome' test in which the internal transcribed spacer1 (situated between the 18S and 5.8S rRNA genes) is amplified. The development of the 'pan-trypanosome' test was a huge improvement in the characterization of trypanosome because it replaced various species-specific PCRs with a single assay that could differentiate the different species by the size of the amplicon. The disadvantage of the test was that it lacked sensitivity and so could not detect trypanosomes in some of the infections, such as those of *T. vivax* (Desquesnes *et al.,* 2001).

Another approach that is useful in distinguishing species of trypanosomes is by analyzing random amplified polymorphic DNA (RAPD). By using a single primer of arbitrary sequence under relaxed PCR conditions, Tibayrenc *et al.* (1993) found a correlation between results that they obtained with multilocus enzyme electrophoresis and RAPD analysis.

PCR is the ideal tool for detection and characterization of trypanosomes because it is more sensitive than the immunological and parasitological techniques and can differentiate parasites that have similar morphologies but different disease outcomes in hosts (Duvallet, 1999; Geysen *et al*., 2003).

2.7 Control of trypanosomosis

For a number of years, efforts have been underway for control of trypanosomosis, with varying degrees of success. Control methods may be directed against the vector or parasite, and may also be directed towards protecting mammalian hosts.

2.7.1 Suppressing the vector

To control tsetse flies, several methods can be employed. Earlier methods involved the use of the Harris trap, killing of wild animals, extensive bush clearing and the use of insecticides. The Harris trap was designed based on the fact that tsetse flies seek for their host using sight,

and would be drawn to the trap due its size. It was found to be efficient when tsetse flies were present in high densities and less efficient when the numbers were low (Du Toit, 1954).

Bush clearing eliminates breeding places for tsetse flies, and is therefore thought to be useful in controlling vector populations (Du Toit, 1954; Okoth, 1999). This would be effective if used in conjunction with other methods of control. The main disadvantage of this approach is that large bushes and trees are cut down, and this is ecologically unacceptable.

Because the presence of wild animals was associated with tsetse flies, the removal thereof was thought to be an effective means for reducing tsetse numbers. The effectiveness of this approach is debatable because in the absence of preferred hosts (wild animals), tsetse flies can feed on other hosts (livestock). In addition, the method usually involves the killing of both infected and non-infected wild animals (Du Toit, 1954; Okoth, 1999).

In 1945 the war office of the United Nations recorded the successful application of Dichloro-Diphenyl-Trichloroethane (DDT) for controlling muscid flies and the vectors of malaria. Thereafter, countries like South Africa embarked on its use. The use of insecticides in South Africa was successful against one of the three species of tsetse flies in KwaZulu-Natal. Although insecticides yield good results, they are discouraged due to concerns about their effects on non-target organisms, and the fact that they can lead to undesirable rise of resistance to insecticides (Mashishi, 2002). Modern insecticides such as deltamethrin are biodegradable, and they can be aerially sprayed at doses that are lethal to tsetse flies but too low to affect other fauna (Mashishi, 2002; Kuzoe and Schofield, 2004).

One of the newly introduced methods of tsetse control is the use of the sterile insect technique (SIT), where male tsetse flies are reared in large numbers, sterilized by irradiation and then released in infested areas. The effectiveness of this technique relies on the fact that the sterile males would mate with wild females resulting in no offspring. Because female flies rarely mate more than once in their lifetime, it is believed that the release of sterile males over a period of time would result in the reduction and perhaps in the complete elimination of tsetse flies. Unlike the other methods of tsetse control, this approach does not have effects on non-target organisms (Kuzoe and Schofield, 2004). SIT is effective in combating tsetse flies when it is integrated with other methods. For example, complete elimination of *G. austeni* was

achieved in 1999 in the Unguja Island of Zanzibar. The success of the campaign relied on the integration of three techniques: reducing tsetse population by capturing them on insecticide impregnated screens and treating cattle with pour-on formulations of pyrethroids from 1988 to 1993, followed by the release of a total of 8.5 million sterile males from 1994 to 1997 (Vreysen *et al*., 2000).

2.7.2 Targeting the pathogen

The second method of trypanosomosis control is one directed against the parasites. This involves the use of drugs for prevention and treatment of trypanosomosis.

Drugs have been used to control trypanosomosis for many decades, but there is a growing concern that the development of resistance in trypanosomes will reduce their effectiveness (Van den Bossche *et al.,* 2006a). Pyrithidium bromide is an effective prophylaxis against *T. vivax* and *T. congolense* infections in sheep, goats and cattle for up to 6 months. Antrycide Prosalt and the newer arsenical cymelarsan give effective protection against *T. b. brucei* infections. The former affords cattle, horses and camels protection for up to three months. Diminazine aceturate (Berenil) and isometamidium chloride are effective against all three livestock-infective trypanosomes. The latter has been used for over 20 years and gives protection for 3-6 months. Resistance to this chemoprophylactic drug has been recorded in East and West Africa (Peregrine, 1994). Homidium bromide has also been found to be an effective chemophrophylactic drug in Kenya. Other chemotherapeutic agents that have been used are the quaternary ammonium trypanocides antrycide, ethidium and prothidium. Trypanocidal drugs are effective for treatment of trypanosome infection but they are expensive and protection is short-lived (Masake *et al.,* 1997).

2.7.3 Use of trypanotolerant livestock

The exploitation of the innate tolerance to trypanosomosis in certain breeds of livestock, some species of wildlife, some strains of mice and possibly humans, is another approach to control trypanosomosis. Trypanotolerance refers to resistance of host animals to trypanosomosis, which appears to be related to their ability to control parasitaemia and resist anaemia. Trypanotolerant animals become infected with trypanosomes, but they do not succumb to disease because they are able to control the multiplication of parasites in their bloodstream.

Because of the limitations of other methods of control, the use of trypanotolerant breeds is accepted as a means of keeping livestock in tsetse-infested areas. When crossbreeds of

N'Dama cattle, which are trypanotolerant, and Nguni cattle, which are not trypanotolerant, were compared, their trypanotolerant trait was found to be intermediate between the two parent breeds and their response to prophylaxis was better than that of their parents (Ordner *et al.,* 1990). The N'Dama and West African Shorthorn cattle are thought to have been in Africa for approximately 7 000 years (Hanotte, 2002).

Both N'Dama and Nguni breeds of cattle are taurine. Since Nguni is not trypanotolerant to the same level as N'Dama, one can infer that Nguni has not been in contact with trypanosomes for a long period of time. Thus, if trypanosomosis is not controlled in South Africa where Nguni is abundant, farmers will always experience great losses of livestock during outbreaks.

2.8 History of tsetse flies and trypanosomosis in KwaZulu-Natal

The incidence of livestock trypanosomosis (*nagana*) in South Africa occurs in the form of waves, in which peaks are followed by long breaks that allow productive livestock farming (Du Toit, 1954).

Nagana in KwaZulu-Natal has always been associated with tsetse flies which were in turn associated with wild animals. In 1879 after game protection laws were enforced in the then Zululand, the number of wild animals increased, accompanied by *nagana* in livestock. To control the *nagana*, three strategies were carried out: the killing of wild animals, trapping of tsetse flies and removal of preferred tsetse habitats. Two major game eradication campaigns were undertaken. The first one took place between 1929 and 1930 whereby more than 37 000 wild animals residing in the Hluhluwe game reserve were killed. The killing stopped in 1930 due to a decrease in the prevalence of *nagana* in cattle. In 1931, 26 000 Harris traps were deployed in the Umfolozi game reserve with the aim of reducing tsetse densities. By 1938, the flies were reduced to a very low figure. Shortly thereafter, tsetse flies began to increase. Even areas that were regarded as "fly free" were found to be infested. This was followed by a widespread outbreak of *nagana*, where more than 60 000 cattle died in the Hluhluwe and Mkuzi settlement areas and in the low-lying eastern portions of the Ngotshe district. This necessitated the second game eradication campaign, which was undertaken from 1942 to 1950. The campaign entailed the killing of more than 130 000 wild animals, accompanied by bush clearing (Du Toit, 1954). *Nagana* reached its highest levels in 1945 despite all the efforts aimed at reducing fly densities by removing sources of blood meal and habitat. Insecticides, DDT and benzene haxachloride (BHC) were considered the next best options for eradicating *G. pallidipes,* then believed to be the principal vector of trypanosomes.

The insecticides were applied by aircraft, released in atomized liquid droplet form and in smoke or thermal aerosol form. The insecticides were also applied with the use of chemical smoke generators and by hand operated bellow-type dusters. Cattle at 144 diptanks were dipped at weekly intervals from 1949 to the end of 1950. The eradication campaign was completed in 1954 and surveys indicated that *G. pallidipes* was totally eradicated from the area (Du Toit, 1954). The cases that were reported thereafter were sporadic and they were diagnosed in cattle, horses and dogs (Kappmeier *et al*., 1998).

In the early 1990s there was a widespread outbreak of trypanosomosis due to infection with both *T. congolense* and *T. vivax* (Kappmeier *et al*., 1998). Cattle at 77 out of 132 diptanks were found to be infected. The disease was effectively managed by treating the cattle with homodium bromide and diminazene aceturate, combined with weekly to fortnightly dipping of cattle in pyrethroid and cyhalothrin (Kappmeier and Nevill, 1999).

Since the outbreak in 1990, little is known on the prevalence of the disease. The incidence and socio-economic impact of *nagana* in KZN remain unknown because it has not been systematically studied and its impact can not be adequately quantified, but is thought to be relatively high (Eisler, 2003).

2.9 Aims and objective of this study

The aim of this project is to contribute to improved understanding of the genetic diversity of trypanosome species infecting cattle at the game/livestock interface in the KwaZulu-Natal Province, South Africa. The specific objectives of this project were to determine:

- The species of trypanosomes in cattle, wild animals and tsetse flies in KZN;
- The genotypic groups of *T. congolense* in cattle, wild animals and tsetse flies in KZN;
- The genetic diversity among Savannah-type *T. congolense* isolates collected in KZN during the period spanning 2005 - 2007.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 The study area

The field study part of this project was carried out in the KwaZulu-Natal Province of South Africa from 2005 to 2007. The analyzed samples were collected from cattle in one commercial farm, Boomerang, and those brought for treatment at 14 geo-referenced diptanks namely: Ekuphindisweni, Mahlambinyathi, Manzabomvu, Mbazwana, Mkhumbikazana, Mpini, Mseleni, Mvutshini, Ndumo, Nhlanjwana, Nhlwati, Nibela, Ocilwane and Qakweni. Samples of wild animals were collected in the Hluhluwe-Umfolozi Game Reserve. Flies were collected from traps set at Boomerang commercial farm, Hluhluwe-Umfolozi, Charter's Creek and Hell's Gate Game Reserves. Location of the 14 diptanks and Boomerang farm are indicated on the map in Figure 3.1. All the above-mentioned locations fall under the Umkhanyakude municipal district, except for Ocilwane, which falls under the Uthungulu district.

Table 3.1: Location of diptanks from where blood samples were collected from cattle.

Name of diptank	Co-ordinate
*Boomerang	S28.13; E32.17
Ekuphindisweni	S26.57;E32.46
Mahlambinyathi	S28.11; E32.19
Manzabomvu	S26.98; E32.25
Mbazwana	S27.51; E32.57
Mkhumbikazana	S27.25; E32.41
Mpini	S27.41; E32.65
Mseleni	S27.36; E32.53
Mvutshini	S28.07; E32.09
Ndumo	S26.93; E32.25
Nhlanjwana	S27.27; E32.24
Nhlwati	S28.00; E31.25
Nibela	S27.85; E32.47
Ocilwane	S28.23; E32.00
Qakweni	S28.08; E32.20

* Commercial farm

3.1.1 General geography

KwaZulu-Natal province is one of the nine provinces of South Africa. It is located in the south-eastern part of the country, and has three geographic areas: the lowland, which is located along the Indian Ocean coast; the central region, which is the Natal Midlands in the undulating hilly plateau and the coastal region which consists of subtropical thickets, deeper ravines and steep slopes. The province covers a total area of 94 361km^2, thus taking up 7.7% of South Africa's land area. KwaZulu-Natal is divided into eleven municipal districts. These are Ugu, Sisonke, Umgungundlovu, Uthukela, Amajuba, Zululand, Umkhanyakude, Uthungulu, Lembe, Umzinyathi and Ethekwini.

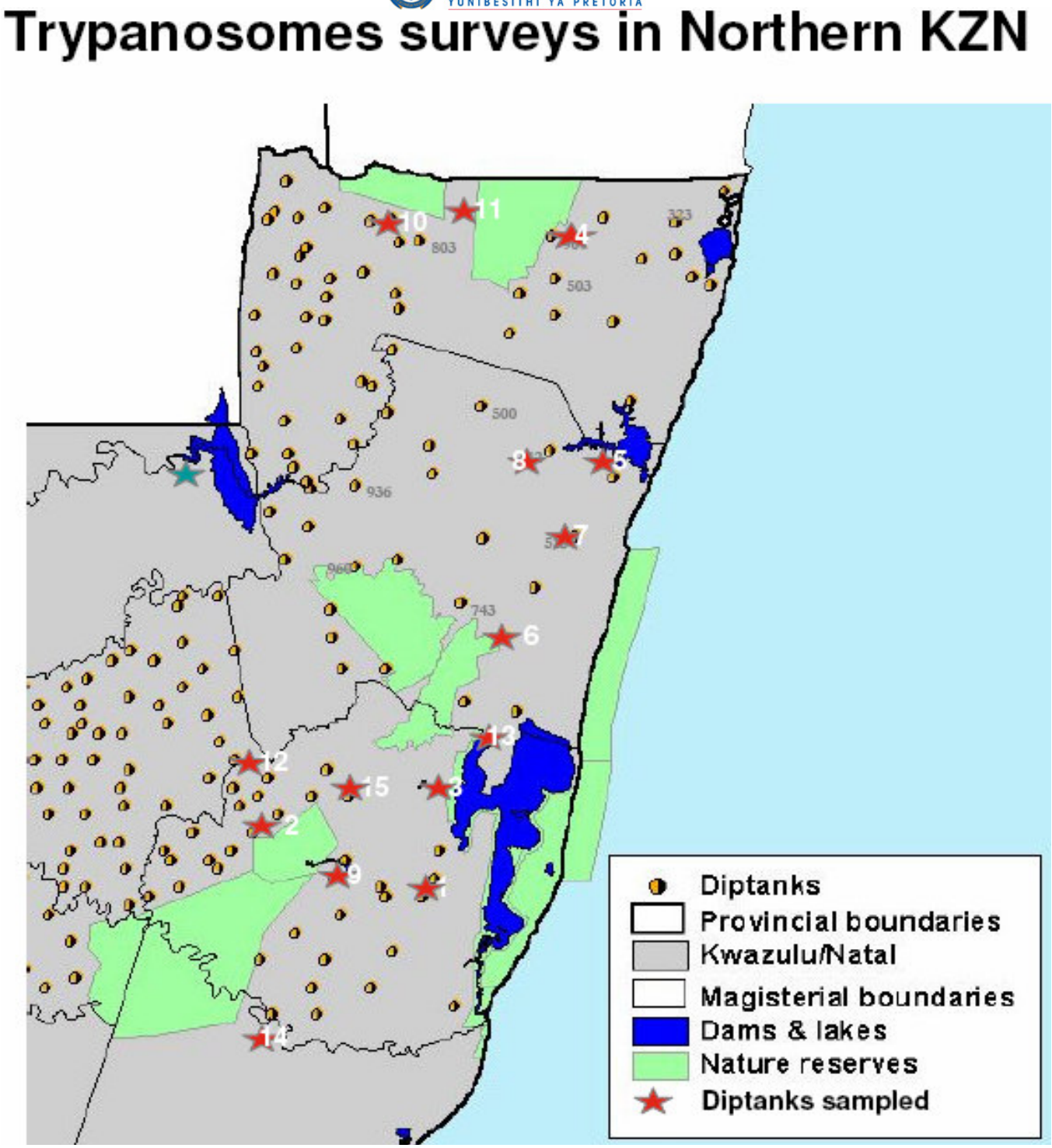

Figure 3.1: A map of the study area. The commercial farm and diptanks are indicated with red stars. The map was compiled and produced by Roy Williams in 2007. Boomerang commercial farm is marked 1, Ekuphindisweni diptank, 2; Mahlambinyathi, 3; Manzabomvu, 4; Mbazwana, 5; Mkhumbikazana, 6; Mpini, 7; Mseleni, 8; Mvutshini, 9; Ndumo, 10; Nhlanjwana, 11; Nhlwati, 12; Nibela, 13; Ocilwane, 14 and Qakweni, 15.

3.1.2 Land use

In rural KwaZulu-Natal, 350 000 to 400 000 households are involved in farming activities. Approximately 58% of agricultural land is used for livestock farming, 17% for crops, 8% for commercial forestry and 3% is set aside for conservation (www.sanbi.org/landdeg/grafics/kwazulunatafs.pdf). The livestock farmers keep a variable mixture of species including cattle, small ruminants, poultry, and to a lesser extent, pigs (Slippers 2001).

3.1.3 Cattle in KZN

3.1.3.1 Breeds

Popular breeds of cattle in KZN are the Brahmans and Nguni. Other breeds such as the Afrikaner, Bonsmara, Drakensberger, Charolais, Hereford, Angus, Simmentaler, Sussex, and Santa Gertrudis are also kept. The cattle are maintained as pure breeds or used in cross breeding (www.kzntopbusiness.co.za).

3.1.3.2 Grazing patterns in communal areas

Cattle in communal areas graze on common fields. Cattle also graze by river beds along the game reserves and on areas where farmers had previously cultivated.

3.1.4 Socio-economic uses of cattle

Cattle are a visible symbol of wealth and status. They are regarded as investments for rural people. The more one has the higher status one would be accorded in the community. In rural communities, they are mostly slaughtered on special occasions such as weddings, traditional rituals and funerals. They are also used in the ploughing of land, kept for milk and beef. Their hides are used to make leather and they are also used to pay *lobola* for *umakoti* (price for a bride). Cattle are also sold when money in cash is required.

3.1.5 Wildlife/livestock interactions-game farming and cattle farming

Direct interaction between livestock and wild animals in KZN does not occur. The game reserves are fenced, and electric fencing is enforced by law if buffaloes are present, and livestock are not permitted in the game reserves. In the drier seasons of the year, cattle belonging to communal farmers graze right up to the fenced boundaries of game reserves, thereby being exposing to tsetse flies that are potentially carrying trypanosomes.

3.2 Sampling

3.2.1 Source of samples

The samples analyzed in this study were collected from sites in KZN known to be infested with tsetse flies. They were taken from 673 cattle, 141 African buffaloes (*Syncerus caffer*), 6 black rhinoceros (*Diceros bicornis*) and 266 tsetse flies.

3.2.1.1 Sampling of cattle

Cattle sampled at communal diptanks are owned by small holder communities that reside next to Hluhluwe Game Reserve and in the districts of Jozini and Ubombo. Cattle at Boomerang belong to a commercial farmer.

Blood was drawn from the tail vein of cattle into a 10ml vacutainer tube containing EDTA as anticoagulant. In Figure 3.2a is a photograph of cattle at Ocilwane diptank lined up in a crush before blood was drawn from some of them. Blood was drawn from sentinel cattle and from the emaciated and sick looking animals brought to the diptanks by their owners. A portion of the blood samples was used in making buffy coats, as described in section 3.3.1.

Figure 3.2a: Cattle lined up in the crush at a diptank in KZN (photograph courtesy of Ms Motloang).

3.2.1.2 Sampling of wild animals

Blood samples were collected from six (rhinoceroses) rhinos which were being relocated from Hluhluwe Game Reserve to Pongola Game Reserve. Figure 3.2b shows how blood was drawn from the ear of a rhino at Hluhluwe-Umfolozi Game Reserve. A total of 141 buffaloes that reside in the Hluhluwe-Umfolozi Game Reserve were also sampled for the purpose of testing them for *Mycobacterium tuberculosis* and trypanosomes. The blood from each buffalo was injected into a mouse and if trypanosomes were present in the blood of the buffaloes, they were allowed to grow to first peak parasitaemia. Blood was then drawn from the parasitaemic mice by cardiac puncture and cryo preserved as stabilates (section 3.2.3.1).

Figure 3.2b: Blood is being drawn from the ear of a rhino at Hluhluwe-Umfolozi Game Reserve (photograph obtained courtesy of Prof Majiwa).

3.2.1.3 Sampling of tsetse flies

Tsetse flies were caught in odour-baited “horizontal” or H traps that were erected at Hluhluwe-Umfolozi, Hells Gate and Charter’s Creek Game Reserve and at Boomerang commercial farm. An extensive description of the H traps is given by Kappmeier (2000). Briefly, the design of the trap is optimized for live collection of two tsetse species that are present in KZN, *G. brevipalpis* and *G. austeni*. The design of the trap resulted from studying the behaviour of the two tsetse species around the Ngu (Ng2f) and Siamese traps. The H trap is made from a pthalogen blue cloth and it’s baited with synthetic odours; octenol (released at *c.* 9.1m/h), 4-methyl phenol (released at 15.5mg/h) and acetone (released at 350mg/h). Unlike previously used traps, the H traps are fitted with two lateral cones. The pthalogen blue cloth was used after studies on coloured targets indicated that *G. brevipalpis* was attracted to that colour. The synthetic odours mimic tsetse host odours, and as such increase the efficiency of the traps (Kappmeier and Nevill, 1999). Lateral fitted rather than vertical cones were included because of the observation that the two species preferred horizontal rather than vertical movement once they had entered traps.

A summary of the location where the traps were set, the number of flies and species examined from each trap is shown in Table 3.2.

To avoid or reduce fly mortality, traps were set in the shade of trees early in the mornings and late in the afternoons. Trapped flies were then collected in the morning and late afternoon, transported to the ARC Tsetse Research Station, Kuleni, KwaZulu-Natal, and kept covered under a dark cloth in the lab before dissection on the same or following day. Before dissection, the flies were immobilized by cooling at 4ºC for approximately 10minutes. Dissection was carried out in phosphate-buffered saline in 2% glucose (PSG) under a dissection microscope. The dissected fly organs (midgut and proboscis) were subsequently placed separately on a microscope slide, a cover slip was placed on the sample and examined at 400x magnification for trypanosomes.

In other cases, the tsetse organs were dissected and the midgut was blotted onto FTA elute cards, whilst proboscides were rinsed in 40µl PSG buffer and also blotted onto FTA elute cards.

Table 3.2: Summary of location of traps, the number of flies caught and species of the tsetse flies.

Location of trap	**Number of flies caught**	**Species**
Hluhluwe-Umfolozi	72	*G. brevipalpis*
Boomerang	78	*G. brevipalpis*
Charter's Creek	76	*G. austeni* and *G. brevipalpis*
Hells Gate	40	*G. brevipalpis*

3.2.2 Expansion of trypanosomes

3.2.2.1 Expansion in cattle and goat

Individual cages containing wild *G. austeni* from Charter's Creek were allowed to feed on the experimental animals with the aim of expanding the trypanosomes that were present in the flies.

A cage containing nine *G. austeni* was placed on the flank of a bovine that was free of trypanosomes. The bovine was kept in insect free stables. Parasitaemia was monitored by microscopic examination of the thin wet smears and buffy coats for motile trypanosomes. The number of parasites per millilitre of blood was calculated by counting the number of parasites per field, converting the number to a logarithmic value and matching it to the table of Herbert and Lumsden (1976). Another cage containing 20 wild *G. austeni* from Charter's Creek Game Reserve were allowed to feed on the ear of an uninfected goat. Parasitaemia in the goat was monitored as described for the bovine.

3.2.2.2 Expansion in mice

Female BALB/c mice were used for expansion of trypanosomes recovered from cattle, wild animals or tsetse flies. The mice were acquired from Onderstepoort Biological Products and maintained at the animal facility within Onderstepoort Veterinary Institute. Occasionally mice were carried to the field, and whenever the buffy coat of an animal was positive for trypanosomes, 0.5ml of the blood was injected intraperitoneally into the mouse. When buffy coats of the rhinos were examined for trypanosomes, they were found to be negative; nevertheless, the blood thereof was injected into mice. As already mentioned in section 3.2.1.1, the blood from 141 buffaloes was injected into mice.

Parasitaemia in mice was monitored three times a week by microscopic examination of the thin wet smears for trypanosomes. Parasitaemia of mice was monitored from day 3 to day 40 after which they were recorded as aparasitaemic if no trypanosomes were seen.

A total of 19 proboscides and one midgut of tsetse flies which were found to be infected with trypanosomes were individually transferred to 0.2ml tubes containing PSG buffer. After thorough suspension, the buffer was injected intraperitoneally into mice. Parasitaemia in the mice was monitored from day 7 to day 40 after which they were recorded as aparasitaemic if no trypanosomes were seen.

3.2.2.3 Expansion in culture

Procyclic cultures were initiated using bloodstream form parasites derived from infected mice. An infected mouse was taken into the lab, its tail was disinfected with 70% ethanol and the tip of the tail was cut-off (approximately 1mm) using sterile scissors. Three drops or 15µl of highly parasitized blood was collected from tip of the tail and inoculated in 1ml of complete MEM culture medium (Table 3.3). Procyclic form trypanosomes were grown from bloodstream forms. Transformation of bloodstream to procyclic forms was carried out by using modified MEM medium containing haemin chloride (0.2mg/100 ml). Once growth was established, procyclic trypanosomes were maintained on the same medium without haemin chloride supplementation. Cultures were maintained at 28ºC in a desiccator where a candle was burned to extinction. The media was changed whenever trypanosomes per well were ~1 x 10^9.

Table 3.3: Complete culture medium for propagation of procyclic trypanosomes.

Compound	**Final concentration**
Foetal bovine serum (heat-inactivated)	20%
HEPES	20mM
L-glutamine	4mM
L-proline	1mM
Hypoxanthine	0.2mM
Penicillin	100IU/ml
Streptomycin	100µg/ml
MEM*	-

*Minimum essential medium with Earle's salts and non-essential amino acids

3.2.3 Storage of trypanosomes

For long-term storage of viable and/or infective trypanosomes, the parasites were frozen in the presence of a cryo protector, glycerol (10%).

3.2.3.1 From blood

To prepare stabilates from the blood of an infected animal, the parasitaemia must be high, i.e., 1 x 10^8 trypanosomes/ml. For preparation of the stabilates, blood was collected from the infected mice and mixed with EDTA, final concentration of 1.5mM. An equal volume of 20% glycerol was added to the blood for a final concentration of 10% glycerol in blood. The stabilates were kept at -80ºC, after 24 hours they were stored in liquid nitrogen.

3.2.3.2 Culture-derived

To prepare stabilates of cultured trypanosomes, the procyclic form trypanosomes were grown until they reached high densities in each well (>1 x 10^9 trypanosomes per ml). The contents of the well were collected and centrifuged at 2 000g for 10minutes. The supernatant was discarded and the pelleted trypanosomes were resuspended in fresh media, to which an equal volume of medium containing 20% glycerol was added for a final concentration of 10% glycerol. The stabilates were frozen and kept at -80ºC.

3.2.4 Resuscitation of trypanosome stabilates

Trypanosome stabilates can be kept frozen for long periods provided there is no breakage in cold chain. When required for inoculation, a tube containing the blood stabilates prepared from an infected mouse was thawed at 37ºC and injected immediately into a mouse.

When the stabilate contained culture forms, it was thawed at 37ºC, centrifuged at 2 000g for 10minutes and the supernatant was discarded. The trypanosome pellet was then resuspended in 1ml of fresh complete MEM and transferred into a well of a 24-well culture plate, maintained at 28ºC in a dessicator and monitored for growth.

3.2.5 Purification of trypanosomes

Trypanosomes grown in rats were separated from blood cells and platelets by passing parasitaemic blood from the infected animals through a column of the anion exchanger, diethylaminoethyl cellulose (DEAE-cellulose) as described by Lanham and Godfrey (1970). Purified trypanosomes were stored at -20ºC.

3.2.6 Trypanosome isolates

The trypanosome isolates analyzed in this study were collected from cattle and buffaloes. Table 3.4 shows all the isolates that were analyzed, the host from which each was isolated and location where the host was sampled. The nomenclature adopted for numbering the isolates is as follows: the first two or three letters represent the institute which provided the isolate. Isolates from the Onderstepoort Veterinary Institute start with OVI, those from the University of Pretoria with UP. KZN is the province from where they were collected. The next letter is B, C or T. B means the isolate was from a buffalo, C from cattle and T from tsetse flies. Another T stands for trypanosomes, and the number following is unique for each isolate. The number /05 or /06 represents the year the isolate was taken from the original host. For instance isolate, OVIKZNCT/4053/06 is an isolate at Onderstepoort Veterinary Institute, originally collected from KwaZulu-Natal province, from cattle number 4053 in the year 2006. Isolates OVIKZNCT/4053/06, OVIKZNCT/8381/06, OVIKZNCT/8394/06 were originally from cattle at Boomerang commercial farm. Blood from those cattle were brought to OVI and injected into other cattle kept in insect-free stables. When parasitaemia in the cattle reached antilog 7.8 /ml, according to the Herbert and Lumsden method, culture was initiated from the blood of each one. Isolates UPKZNCT/3/05, UPKZNCT/5/05, and UPKZNCT/11/05 were from cattle at Mvutshini diptank. Blood from these cattle was injected into BALB/c mice. At peak parasitaemia the trypanosomes were made into stabilates and stored in liquid nitrogen. The stabilates were later resuscitated and injected into mice. When parasitaemia in the mice was antilog 7.8/ml, culture was initiated.

Table 3.4: Trypanosome isolates analyzed in the study.

OVIKZNCT/4053/06	Bovine	Boomerang
OVIKZNCT/8381/06	Bovine	Boomerang
OVIKZNCT/8394/06	Bovine	Boomerang
UPKZNCT/3/05	Bovine	Mvutshini
UPKZNCT/5/05	Bovine	Mvutshini
UPKZNCT/11/05	Bovine	Mvutshini
UPKZNBT/1/06	Buffalo	Hluhluwe
UPKZNBT/2/06	Buffalo	Hluhluwe
UPKZNBT/3/06	Buffalo	Hluhluwe
UPKZNBT/4/06	Buffalo	Hluhluwe

3.3 DNA

3.3.1 Applying samples onto FTA cards (Whatman®)

Buffy coats were made from a portion of blood samples collected from animals (cattle, wild animals and mice). To make buffy coats, blood was either drawn from the tail of a mouse or already in a vacutainer tube (from cattle and wild animals) was drawn by capillary action into haematocrit tubes (diameter 1.5mm), then centrifuged for 3minutes at 11 000g in a micro haematocrit centrifuge, to separate plasma from blood cells. A buffy coat forms at the interface of packed cells at the lower part and serum at the top part. The capillary tube was cut just above the packed red blood cells with a diamond pencil then immediately placed on a filter paper; and the buffy coat (where trypanosomes become concentrated) flowed from the tube onto the FTA filter paper by capillary action.

Pelleted and non-pelleted cultured trypanosomes were applied to FTA elute cards. To pellet the trypanosomes, 1ml of the culture medium was placed in a 1.5ml tube, centrifuged for 10minutes at 11 000g and the supernatant was discarded. The pellet was resuspended in 40µl of nuclease free water and applied to an FTA elute card.

For non-pelleted trypanosomes, 40µl of the cultures were applied directly onto FTA elute cards. All samples applied on FTA cards were labelled and allowed to dry at room temperature and stored, making sure there is no possibility of cross-contamination.

3.3.2 Extraction of genomic DNA

To extract genomic DNA, cultured trypanosomes were pelleted by centrifugation at 7 000g for 10minutes. The pellet was then resuspended in NET (150mM NACl, 0.1mM EDTA and 10mM Tris, pH 7.4) at about 1 x 10^8 trypanosomes/ml. To lyse the cells, 10% SDS was added to a final concentration of 1%, followed by incubation at 37ºC for 10minutes to solubilize and disperse the cells. RNAse A (10mg/ml) was then added to a final concentration of 100µg/ml and the solution was further incubated at 37ºC for 2hours. Proteinase K (10mg/ml) was then added to a final concentration of 100µg/ml and incubated for an additional 2hours. Extraction was performed with NET saturated-phenol, and centrifuged at 2 000g for 10minutes to separate the phases. The aqueous phase was transferred to a clean eppendorf tube and extracted twice, sequentially, each time with an equal volume of water-saturated phenol. The aqueous phase was then recovered into a clean tube, and extracted with an equal volume of

diethyl ether. To precipitate DNA, 2.5x volume of cold (-20ºC) absolute ethanol was added to the aqueous phase and mixed gently. The solution was then incubated overnight at -20ºC. The DNA was recovered by centrifugation at 11 000g for 30minutes at 4ºC. The supernatant was discarded and the pellet was washed in 70% ethanol, after which it was vacuum-dried and resuspended in 100µl of nuclease free water. The extracted DNA was either used immediately or stored at -20ºC.

3.3.3 Preparation of samples for PCR

3.3.3.1 From FTA classic cards

To prepare samples placed on FTA classic cards for PCR, 2.0mm disks were punched from where samples were applied, and washed following the manufacturer's instructions (Whatman, Biosciences Ltd, Brentford, U.K.). Briefly, the disks were washed three times, each time with 300µl of FTA purification reagent (Whatman). This was followed by three consecutive rinses in 300µl of TE-1 buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) The washed disks were transferred to clean tubes, placed in a heating block at 55ºC until they were dry. The disks were then considered ready for use in PCR amplification.

3.3.3.2 From FTA elute cards

To prepare samples that were placed on FTA elute cards for PCR amplification, 3mm disks were punched from where the samples were applied and washed following the manufacturer's instructions (Whatman, Biosciences Ltd, Brentford, U.K.). Briefly, the disks were washed in 500µl of nuclease free H_2O by pulse vortexing tubes three times for a total of 5seconds. The disks were then transferred to 0.5ml eppendorf tubes containing 30µl of nuclease free H_2O. To ensure that the disks were completely immersed, the tubes were centrifuged at 11 000g for 10seconds, after which they were incubated at 95ºC for 30minutes. At the end of the incubation period, the tubes were centrifuged at 11 000g for 30seconds, to separate the disks from eluates containing DNA. The disks were then removed and the eluates were either used immediately in amplification reactions or stored at -20ºC.

3.3.4 Gel clean-up

Prior to digestion, bands from the semi-nested amplification were excised from the agarose gel using a sharp scalpel blade. The PCR product was then purified using the QIaquick Gel

Extraction kit (Qiagen) following the manufacture's instructions. The purified PCR product was either used immediately or stored at -20ºC.

3.4 Oligonucleotide primers

Oligonucleotide primers were purchased from Inqaba Biotechnical Industries (PTY) Ltd (Pretoria, RSA). The DNA sequence of the primers and the expected product sizes are listed in Table 3.5.

Primers designated 18ST nF2, 18ST nR2 and 18ST nR3 target the 18S ribosomal RNA gene of trypanosomes. In Figure 3.3 is a depiction of the structure of the 18S ribosomal RNA gene locus, and the relative positions and direction of the primers.

Primers designated TBR, TCN, TCK and TVM are specific for *T. brucei,* Savannah-type *T. congolense*, Kilifi-type *T. congolense* and *T. vivax* respectively. Primers designated ILO509 and ILO524 are of arbitrary nucleotide sequence and were used in RAPD analysis.

Figure 3.3: A diagram illustrating a generalized structure the 18S ribosomal RNA gene locus in trypanosomatids. The diagram is based upon the sequence of *T.congolense* IL1180 ribosomal RNA gene, GenBank ACCESSION U22315. The direction and locations of primers used in the study are indicated. 18ST nF2 and 18ST nR3 are used in the first amplification and 18ST nF2 and 18ST nR2 are used in the nested amplification.

Table 3.5: Oligonucleotide primers for amplification of DNA from different trypanosomes.

Specificity of primers	Forward primer	Reverse primer	Expected product size (bp)	Literature reference	GenBank reference no
African trypanosomes	5'- CAA CGA TGA CAC CCA TGA ATT GGG GA- 3' **(18ST nF2)**	5'-TGC GCG ACC AAT AAT TGC AAT AC- 3' (**18ST nR3**)	750	Geysen *et al.* 2003	U22315, U22316, U22317
African trypanosomes	5'- CAA CGA TGA CAC CCA TGA ATT GGG GA- 3' **(18ST nF2)**	5'-GTG TCT TGT TCT CAC TGA CAT TGT AGT G- 3' **(18ST nR2)**	650	Geysen *et al.* 2003	U22315, U22316, U22317
T. brucei	5'- CGA ATG AAT ATT AAA CAA TGC GCA GT- 3' **(TBR1)**	5'- AGA ACC ATT TAT TAG CTT TGT TGC- 3' **(TBR2)**	177	Sloof *et al.* 1983a	K00392
T.congolense Kilifi	5'- GTG CCC AAA TTT GAA GTG AT-3' **(TCK1)**	5'-ACT CAA AAT CGT GCA CCT CG-3' (**TCK2**)	294	Masiga *et al.*, 1992	S50875
T.congolense Savannah	5'- TCG AGC GAG AAC GGG CAC TTT GCG A- 3' **(TCN-1)**	5'-ATT AGG GAC AAA CAA ATC CCG CAC A- 3' **(TCN-2)**	341	Majiwa and Otieno, 1990	M30391
T.vivax	5'- TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG- 3' **(TVMF)**	5' CAG CTC GGC GAA GGC CAC TTG GCT GGG GTG- 3' **(TVMR)**	400	Masake *et al.*, 1997	U43183
Not specific	5'- TGG TCA GTC A- 3' **(ILO509)**	None			N/A
Not specific	5'- CGC GCC CGC T- 3' **(ILO524)**	None			N/A

3.5 Analyses

3.5.1 Microscopy

Buffy coats were prepared from blood collected from animals, then placed on microscope slides and examined for presence of motile trypanosomes at 400x magnification. The number of trypanosomes per ml was estimated according to the matching method of Herbert and Lumsden (1976). Microscopy at 400x magnification was also used to examine the proboscides and midguts of tsetse flies.

3.5.2 Amplification reactions

Three types of amplification reactions were carried out in this study; trypanosome specific, species-specific and random amplification.

To amplify trypanosome DNA, two rounds of amplification were employed in a semi-nested protocol designed by Geysen *et al.* (2003). In the first round of amplification, 18ST nF2 was used as the forward primer and 18ST nR3 as the reverse primer. For the second round of amplification, 18ST nF2 (also used in the first amplification) was used as the forward primer and 18ST nR2 as the reverse primer. Product from the first round was used as template for the second round. The first amplification reaction was carried out in a final volume of 50µl or 25µl (FTA classic or elute cards respectively) containing 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 200µM of each dNTP, 0.4µM of each of the primers and 0.5U *ExTaq* polymerase (Takara). Positive (10ng of reference DNA) and negative (no DNA) controls were included in each set of experiments.

In instances where samples were applied onto FTA classic cards, a washed disk was placed in 50µl of the PCR mix. When FTA elute cards were used, 2.5µl of the eluted DNA was added to 22.5µl of the PCR mix. The reaction tubes were placed in a thermal cycler, incubated at 94ºC for 4minutes in an initial denaturation step, followed by 40 cycles of 1min at 94ºC, 1.5min at 58ºC and 2min at 72ºC. At the end of the cycles, the samples were cooled to 4ºC until they were removed from the cycler.

The second amplification reaction was carried out in 25µl. The reaction mix consisted of 24.5µl of the PCR mix (same as for the first round except for the reverse primer) and 0.5µl of product from the first amplification. The reaction tubes were placed in a thermal cycler and incubated at 94ºC for 4minutes in an initial denaturation step, followed by 25 cycles of 1min at 94ºC, 1.5min at 58ºC and 2min at 72ºC. The samples were then cooled to 4ºC until they were removed from the cycler.

To amplify a species-specific product, primers that are specific for *T. brucei*, *T. vivax*, Savannah-type *T. congolense* or Kilifi-type *T. congolense* respectively were used. The amplifications were carried out in 50µl or 25µl (FTA classic or elute cards respectively) reaction volumes containing 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 100µM of each dNTP, 0.2µM of each of the primers and 0.5U *ExTaq* polymerase (Takara). Positive reference DNA and negative (with non-target and without DNA) controls were included in each set of experiments. The proboscides and midguts of colony flies (reared at Onderstepoort Veterinary Institute) were also used as negative controls. When samples were applied onto FTA classic cards, the washed disk was placed in 50µl of the PCR mix. When FTA elute cards were used, 2.5µl of the eluted DNA was added to 22.5µl of the PCR mix, and placed in a thermal cycler.

For *T. vivax* specific amplification, tubes were incubated at 94ºC for 4min in an initial denaturation step, followed by 30 cycles of 1 min at 94ºC, 1 min at 65ºC and 1min at 72ºC. The samples were then cooled to 4 ºC until they were removed from the cycler.

For *T. brucei*, Savannah-type *T. congolense* and Kilifi-type *T. congolense*, tubes were incubated at 94 ºC for 4 min in an initial denaturation step, followed by 30 cycles of 1min at 94ºC, 1min at 60ºC and 1 min at 72ºC. The samples were then cooled to 4ºC until they were removed from the cycler.

PCR amplification using RAPD primers was carried out in 25µl reaction volumes containing 50ng of trypanosome genomic DNA, 10mM Tris-HCl, pH 8.3, 50mM KCl, 3.0mM $MgCl_2$, 200µM of each dNTP, 0.6µM of primer and 2.5U *ExTaq* polymerase (Takara). The reaction tubes were incubated in a thermal cycler programmed for 30 cycles of 94ºC for 1min, 40ºC for 1.5min, 72ºC for 2min followed by a 5min extension at 72ºC. The samples were then cooled to 4ºC until they were removed from the cycler.

3.5.3 RFLP

Nested PCR products were double digested with restriction enzymes, *Msp*I and *Eco*571 in buffer Y+/Tango with S-adenosylmethionine according to specifications given by the manufacturer (Fermentas). The digestion was carried out at 37ºC overnight and inactivated at 65ºC for 20minutes.

3.5.4 Gel electrophoresis

All PCR products were electrophoresed through agarose gels that were stained with ethidium bromide. Products from amplification of the rRNA gene were electrophoresed through 1.5% agarose in 1x TAE buffer at 120V for 1hour. Products from species-specific amplification were electrophoresed through 1.5% agarose in 1x TAE buffer at 120V for 30minutes and RAPDs were electrophoresed through a 0.7% gel for 4hours. The GeneSnap from SynGene software was used to visualize and capture gel images.

Products from amplification of the rRNA gene that were digested with restriction enzymes were electrophoresed through 10% polyacrylamide gel in 1x TBE buffer at 100V for 2.5hours. The gel was stained using a commercial silver staining kit (BIORAD).

4. RESULTS

4.1 Optimized PCR conditions

4.1.1 Specificity of ribosomal RNA gene primers

The specificity of ribosomal rRNA gene primers 18ST nF2, 18ST nR2 and 18ST nR3 was evaluated in PCR using as targets DNA of *T. brucei* A4 ILTat 1.1, Savannah-type *T. congolense* IL3000*, T. vivax* IL2160, *Theileria parva*, *Babesia bigemina* and *Babesia bovis.* These primers were shown to be specific and no amplification was detected with non-trypanosomatid DNAs in the first and in the semi-nested amplification (data not shown).

PCR products of reference DNA are shown in Figure 4.1. Products from the first and the semi-nested amplification are loaded in adjacent lanes. The expected products (based on sequences in GenBank) from the first round of amplification are; 765bp for *T. brucei*, 770bp for Savannah-type *T. congolense* and 710bp for *T. vivax*. The obtained products from the first round of amplification were; 770bp for *T. brucei*, 760bp for Savannah-type *T. congolense* and 720bp for *T. vivax*. The expected products from the semi-nested amplification are; 658bp for *T. brucei*, 669bp for Savannah-type *T. congolense* and 613bp for *T. vivax*. The obtained products from the semi-nested amplification were; 660bp for *T. brucei*, 660bp for *T. congolense* and 620bp for *T. vivax*.

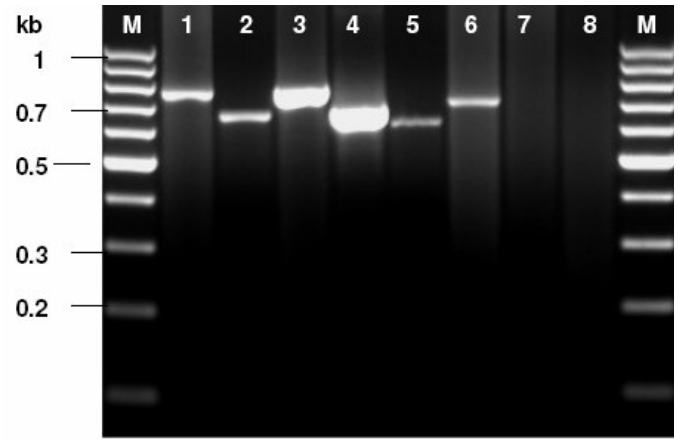

Figure 4.1: Products from PCR amplification using 18ST nF2 with 18ST nR3 primers and 18ST nF2 with 18ST nR2 primers on purified DNA of: *T. brucei* A4 ILTat 1.1, in lanes 1 and 2; *T. congolense* IL3000, lanes 3 and 4; T.vivax IL2160 lanes, 6 and 5, respectively and no target DNA (H_2O), in lanes 7 and 8. Lanes 1, 3, 6 and 7 contain products from the first round of amplification, whereas lanes 2, 4, 5 and 8 contain products from the semi nested amplification. Lane M contains the O'GeneRuler 100bp DNA ladder (Fermentas).

PCR products from the semi-nested amplification were double digested with *Msp*I and *Eco*571, and RFLP profiles that differentiate *T. brucei*, *T. congolense* and *T. vivax* were obtained. The expected band sizes for the three species of trypanosomes are summarized in Table 4.1. They were predicted based on sequences of the ribosomal RNA genes deposited in GenBank. Bands of sizes; 113, 137, 171 and 182bp are characteristic of *T. brucei.* Other 2 bands of sizes; 24 and 31bp migrate out of the gel and are therefore not visualized. Bands of sizes; 160, 189 and 280bp are characteristic of Savannah-type *T. congolense.* A 40bp band migrates out of the gel and is therefore not visualized. *T. vivax* is characterized by presence of 124, 198 and 244bp bands. A 47bp band migrates out of the gel. The data is shown in Figure 4.2.

Table 4.1: Expected band sizes when PCR products obtained using ribosomal primers (18ST nF2 and 18ST nR2) are double-digested with *Msp*I and *Eco*571.

Species	**Accession no.**	**Fragment 1**	**Fragment 2**	**Fragment 3**	**Fragment 4**
T. brucei brucei	AB301937	113	137	171	182
T. congolense	U22315	160	189	280	
T. vivax	U22316	124	198	244	

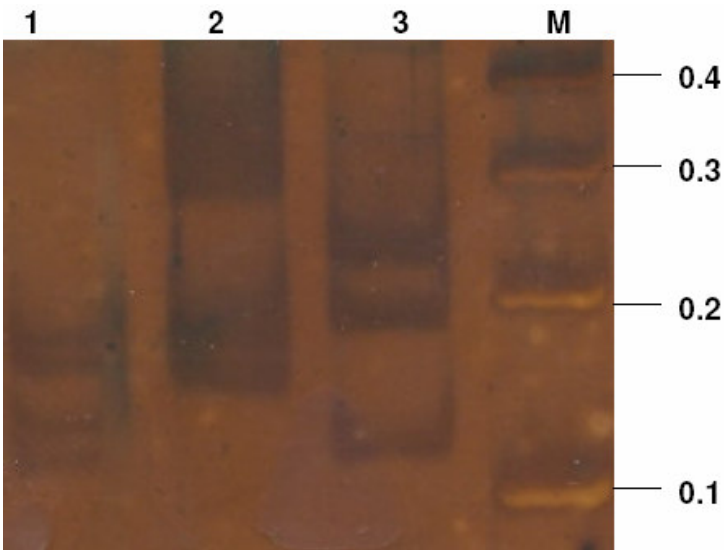

Figure 4.2: RFLP profiles generated upon double digestion (with *Msp*I and *Eco*571) of PCR products obtained using 18ST nF2 and 18ST nR2 primers. In lane 1 is digests of *T. brucei*; lane 2, *T. congolense*; lane 3, *T.vivax*. Lane M contains the O'GeneRuler 100bp DNA ladder (Fermentas).

4.1.2 Specificity of trypanosome species-specific primers

Various primers that target DNA sequences present only in certain species of trypanosomes have been discovered. Such primers are useful in the specific identification of different trypanosome species. Before applying these primers in amplification of DNA that may be present in samples collected from the field, conditions under which they had to be used were optimized. This was done using target and non-target DNA. For example, in optimizing TBR primers, which are specific for *T. brucei,* DNA of *T. brucei* (target DNA), Savannah-type *T. congolense* and *T. vivax* (non-target DNAs) were used. These tests were carried out for *T. brucei*, Savannah-type *T. congolense*, Kilifi-type *T. congolense* and *T. vivax* specific primers. Amplification conditions were optimized such that no PCR products were obtained from non-target DNAs in each case.

The use of TBR primers under optimal conditions resulted in a 177bp product from *T. brucei* DNA. TVM primers gave a 435bp product from *T. vivax* DNA (Figure 4.3, panel A, lane 3). TCN primers resulted in a 320bp product from the DNA of Savannah-type *T. congolense* (Figure 4.3, panel B, lane 2). The use of TCK primers resulted in a 294bp product for Kilifi-type *T. congolense.*

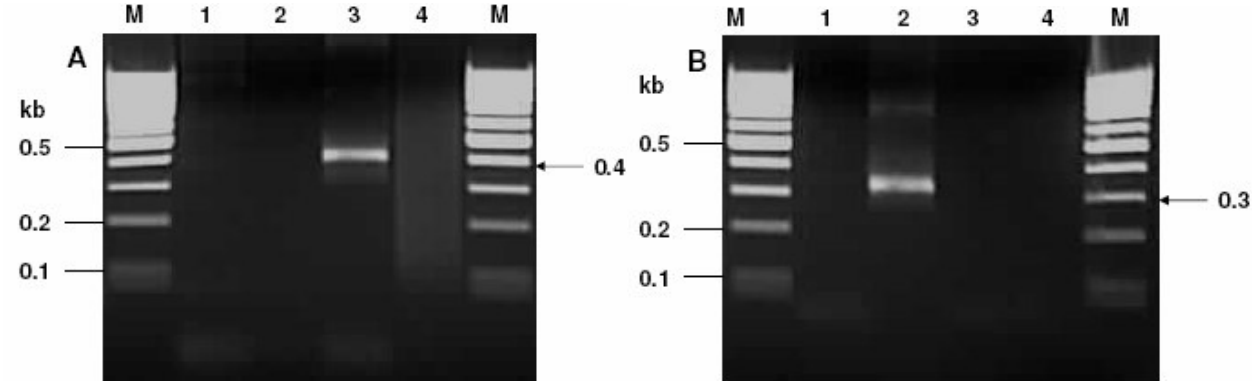

Figure 4.3: Species-specific amplification using *T. vivax* specific primers in panel A and Savannah-type *T. congolense* specific primers in panel B. In both panels, the PCR products loaded were obtained by amplification of DNA purified from: (1) *T. brucei* A4 ILTat 1.1; (2) *T. congolense* IL3000; (3) *T.* vivax IL2160; (4) water (no target DNA) and lanes M contain the O'GeneRuler 100bp DNA ladder (Fermentas).

4.1.3 FTA elute cards are superior

Initially, PCR amplifications were carried out on DNA samples that were extracted with the automated Magna Pure LC system (Roche, Germany). Amplification of samples that were microscopically positive yielded no PCR product when ribosomal RNA gene, Savannah-type *T. congolense* and *T. vivax* specific primers were used. To determine whether buffers used for DNA extraction were inhibiting the PCR, the reaction was spiked with DNA purified from *T. congolense* IL3000. The reaction mix consisted of the extracted DNA, 0.09ng positive control DNA, dNTPs, buffer, *Taq* polymerase and ribosomal RNA gene primers; 18ST nF2 with 18ST nR3 (first round amplification), and 18ST nF2 with 18ST nR2 in the semi-nested amplification. An expected PCR product was obtained in the spiked sample, indicating that there was nothing inhibiting the reaction, or else *T. congolense* purified DNA would not have amplified.

To determine if host DNA was inhibiting the PCR, the extracted DNA was diluted down from 10µl of extract in 50µl reaction volume to 0.5µl extract in 50µl reaction volume. Still, no amplification occurred.

When the buffy coats of the same microscopically positive samples were placed on Whatman FTA classic cards (Whatman, UK) and used in PCR, an expected product was obtained (data

not shown). Henceforth, extraction of samples using the Magna Pure LC system was stopped, and all the samples were applied onto Whatman classic cards for further processing. The use of FTA classic cards was limiting because only one reaction could be carried out per disk cut out of the filter onto which the sample had been applied. Furthermore, it was also doubtful whether the washing of the FTA disks was sufficient to remove all PCR inhibitors such as haemoglobin. The Whatman classic cards were then compared with Whatman FTA elute cards (Whatman, UK). When the same amount of sample was placed on the FTA classic card and FTA elute cards, washed or eluted respectively, and used in PCR, amplification of DNA from FTA elute cards was better than that from FTA classic cards (Figure 4.4), as reflected by the intensities of the amplicons. FTA elute cards were selected as the preferred DNA storage matrix over FTA classic cards.

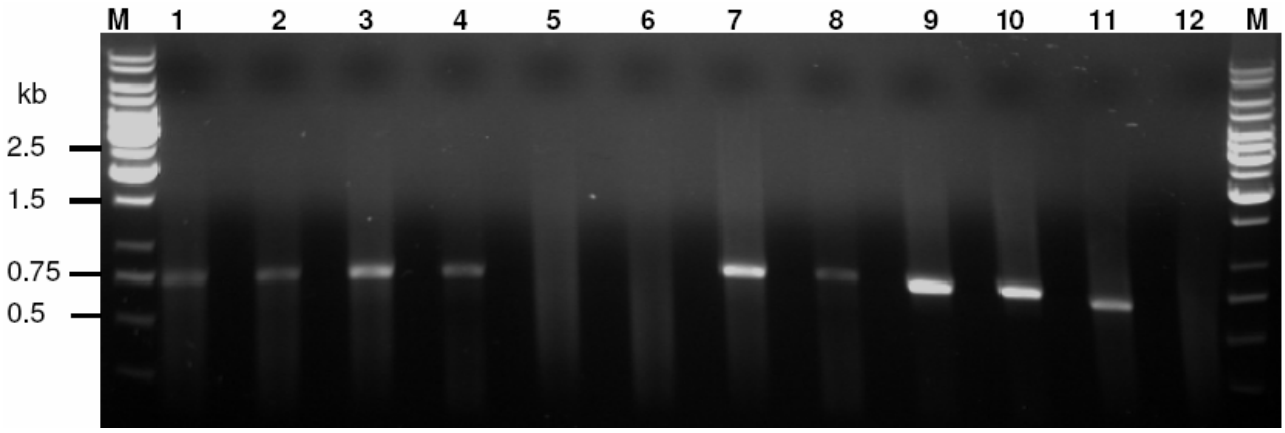

Figure 4.4: Products from PCR amplification using ribosomal RNA gene primers (18ST nF2 and 18ST nR3) on samples that were stored on two different FTA matrices. Amplicons that are loaded in lanes 1, 3, 5 and 7 were from samples that were stored on FTA elute cards; in lanes 2, 4, 6 and 8 are samples that were stored on FTA classic cards; lane 9, *T. brucei*; lane 10, *T. congolense;* lane 11, *T. vivax* and lane 12 had water. Products obtained from identical samples were loaded in lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8. Lanes M contain the O'GeneRuler 1kb DNA ladder (Fermentas).

4.2 Trypanosomes in cattle

The conditions for use of the primers and the method that led to DNA extracts suitable for PCR were optimized (section 4.1). This was followed by examination of samples collected from the field for presence of different species of livestock-infective trypanosomes.

The total number of samples that were analyzed from cattle included 590 from 14 communal diptanks around Hluhluwe-Umfolozi Game Park and 83 from Boomerang commercial farm. Infection in cattle was determined by PCR using ribosomal RNA gene primers, RFLP analyses and/or PCR with species-specific primers as necessary.

A summary of the results obtained are given in Tables 4.2 and 4.3. Overall, at communal diptanks, 91 of the cattle were positive for trypanosomes: 50 were positive for *T. congolense,* 36 for *T. vivax* and five had mixed infections of *T. congolense* and *T. vivax.* At the Boomerang farm, 29 of the weaners and five of the adult cattle were positive for *T. congolense*.

Table 4.2: Summary of samples collected from cattle at different diptanks and results obtained by PCR analysis.

Name of diptank	**Date of sampling**	**No. of samples**	**No. of +ve samples (%)**	**Trypanosome species detected**
Ekuphindisweni	Nov 2005	38	2 (5.2)	*T. vivax, T. congolense*
Mahlambinyathi	Nov 2005	60	39 (65)	*T. vivax, T. congolense*
Mvutshini	Nov 2005	36	33 (91)	*T. congolense*
Nhlwati	Nov 2005	45	5 (11)	*T. vivax*
Qakweni	Nov 2005	56	4 (7)	*T. congolense*
Ocilwane	Oct 2006	33	0	-
Nibela	Dec 2006	21	0	-
Manzibomvu	May 2007	50	0	-
Mbazwana	May 2007	49	0	-
Mkhumbikazane	May 2007	49	0	-
Mpini	May 2007	16	0	-
Mseleni	May 2007	51	0	-
Ndumo	May 2007	43	4 (9.3)	*T. congolense*
Nhlajwane	May 2007	44	4 (9.1)	*T. congolense*

Table 4.3: Summary of analysis of samples that were collected from cattle at Boomerang commercial farm.

Size	Date of sampling	No. of samples	No. of +ve samples (%)	Trypanosome species
Weaners	Jan 2007	43	29 (67)	*T. congolense*
Adults	Jan 2007	40	5 (12.5)	*T. congolense*

Figure 4.5 illustrates results obtained when ribosomal RNA gene primers (18ST nF2 and 18ST nR3) were used in PCR on 10 samples collected from the weaners. In lanes 1, 2, 3 and 6 no product was obtained. A smear is seen in lanes 1, 2, and 3 which could be attributed to a non-specific reaction. Lanes 4, 7, 8 and 9 have a product similar in size to that in lane 11 (containing the positive control, *T. congolense* IL3000), indicating that those samples have *T. congolense*. In addition to having products of the same size, lanes 11 (containing the positive control, *T. congolense*) and 9 have a faint band of approximately 1.5kb that is not present in the other lanes. Lanes 5 and 10 have a product that is bigger in size than those in the other lanes, indicating presence of either a different species or genotypic group of trypanosomes.

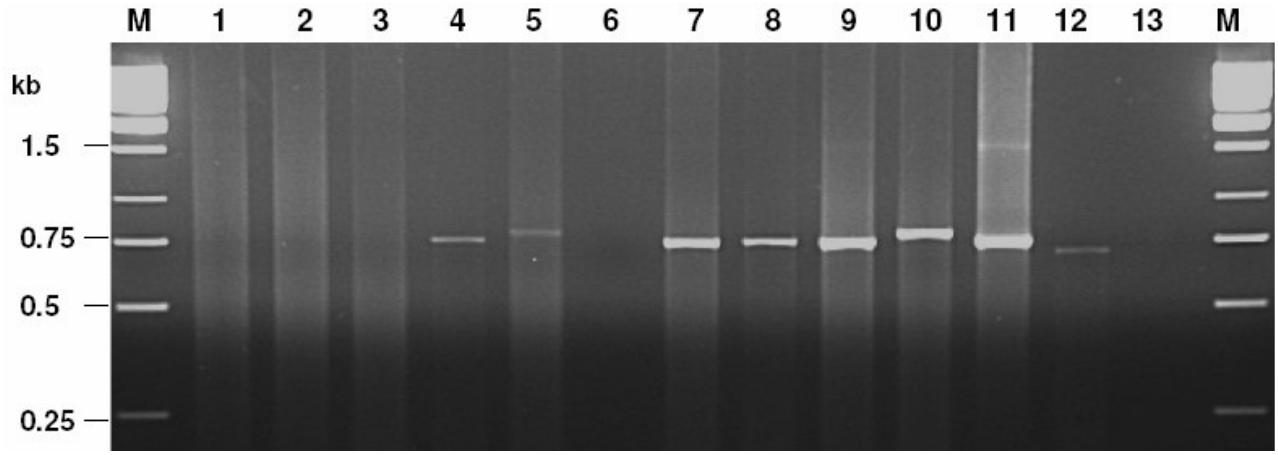

Figure 4.5: PCR products from amplification of DNA eluted from FTA elute cards on which were placed buffy coats from cattle. The primers used (18ST nF2 and 18ST nR3) target the 18S ribosomal RNA gene. In lanes 1-10 are PCR products obtained from buffy coats of animals numbered 1-10; lane 11, *T. congolense* IL3000; lane 12, *T. vivax* IL2160; lane 13, water (no target DNA) and lanes M contain O'GeneRuler 1kb DNA ladder (Fermentas).

Figure 4.6 illustrates PCR results obtained when Savannah-type *T. congolense* specific primers were used on the same samples shown in Figure 4.5. A product of 320bp is present in lanes 4, 6, 7, 8 and 9. Lane 9 has an additional product of approximately 700bp not seen in

any of the other lanes. Savannah-type *T. congolense* specific primers amplified the sample in lane 6 which ribosomal primers had failed to amplify.

Based on PCR results that were obtained using the two sets of primers, seven of the 10 samples were positive for trypanosomes; six revealed by rRNA gene primers (Figure 4.5) and an additional one revealed by species-specific primers (Figure 4.6, lane 6).

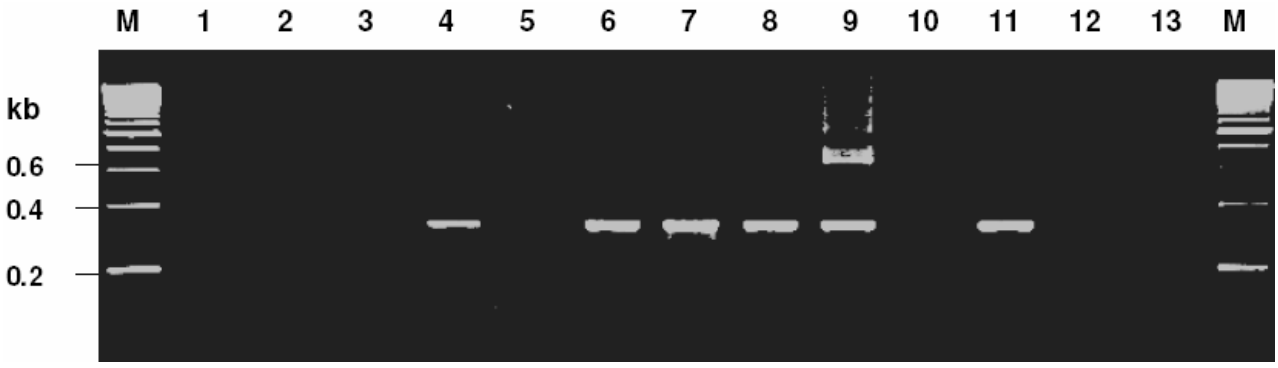

Figure 4.6: PCR products from amplification of DNA eluted from FTA elute cards on which were placed buffy coats from cattle. The primers used are specific for Savannah-type *T. congolense.* In lanes 1-10 are PCR products obtained from buffy coats of animals numbered 1-10; lane 11, Savannah-type *T. congolense* IL3000; lane 12, *T. vivax* IL2160; lane 13, water (no target DNA) and lanes M contain the O'GeneRuler 200bp DNA ladder (Fermentas).

4.3 Trypanosomes in wildlife

When relocating wildlife for conservation or for any other purpose, it is often necessary to determine if they have any pathogenic organisms that they might introduce into their new location. It was possible to obtain blood samples from rhinos that were being relocated from Hluhluwe-Umfolozi to Pongola Game reserve, and trypanosomes from buffaloes residing in the Hluhluwe-Umfolozi Game Reserve.

Only five out of 141 mice that were individually injected with blood from 141 buffaloes became parasitaemic. When the buffy coats of the mice were subjected to PCR using ribosomal RNA gene primers, they were found to be positive for *T. congolense.* In addition, a 320bp PCR product was obtained when the samples were amplified with Savannah-type *T. congolense* specific primers.

As already mentioned, a similar exercise was attempted in rhinos. Mice that were injected with the blood of six rhinos did not become parasitaemic throughout the study. In addition, no PCR product was obtained when either the ribosomal, Savannah-type *T. congolense* specific or *T. vivax* specific primers were used, indicating that the rhinos were free of detectable trypanosomes at the time they were sampled.

4.4 Trypanosomes in tsetse flies

Determination of trypanosomes that tsetse flies were harbouring was undertaken using PCR in combination with microscopy. Two batches of flies were dissected. The first batch consisted of 140 flies, caught in the Hluhluwe-Umfolozi and Charter's Creek Game Reserves and at Boomerang farm. The second batch consisted of 126 flies from Charter's Creek, Hell's Gate and Hluhluwe-Umfolozi Game Reserves. Results from analysis of the first batch of flies are reported here, the other results are given in section 4.6.

Twenty eight flies were caught from traps that were set at Boomerang. Of these, 27 were *G. brevipalpis* and one was *G. austeni*. Of those flies, six (21%) were found to be positive for trypanosomes by microscopy.

At Charter's Creek Game Reserve, 40 flies were caught. Thirty-two were *G. brevipalpis* and eight *G. austeni*. Trypanosomes were seen in six (15%) of the dissected flies.

A total number of 72 flies were caught in traps set within the Hluhluwe-Umfolozi Game Reserve, and all the flies were *G. brevipalpis*. Only eight (11%) of the flies were positive for trypanosomes by microscopy. Data from analysis of flies from three sites are summarized in Table 4.4.

No Parasites were seen in the 20 mice that were injected with the trypanosome positive tsetse organs. However, the buffy coats of all the mice were positive when Savannah-type *T. congolense* specific primers were used in PCR. No PCR product was obtained when primers that target the ribosomal RNA gene were used. The semi nested amplification also resulted in no PCR product (data not shown).

The goat and the bovine on which *G. austeni* from Charter's Creek were fed became parasitaemic. The parasites were identified as *T. congolense* by PCR.

Table 4.4: Summary of flies that were caught in traps at different locations. The flies were dissected and examined by microscopy for presence of trypanosomes.

Location of trap	Date of sampling	Species of flies	No. of flies dissected	No. of +ve organs (%)	Trypanosome species
Boomerang	Oct 2006 Sep 2007	*G. austeni* *G. brevipalpis*	1 27	1 (100) 5 (18.5)	*T. congolense*
Charter's Creek	Oct 2006 Sep 2007	*G. austeni* *G. brevipalpis*	8 32	3 (37.5) 3 (9.4)	*T. congolense*
Hluhluwe-Umfolozi	Oct 2006	*G. brevipalpis*	72	8 (11.1)	*T. congolense*

4.5 Different genotypic groups of *T. congolense* in KZN

In the course of this investigation, PCR amplifications conducted with rRNA gene primers resulted in two size categories of PCR products for *T. congolense*: 690bp (Figure 4.7, lanes 1-7) and 705bp (Figure 4.7, lanes 8 and 9). The 690bp PCR product corresponds to that obtained with the Savannah-type *T. congolense* DNA (Figure 4.7, lane 10). The PCR product of 705bp corresponds most closely to what is expected from this locus in the Kilifi-type *T. congolense*.

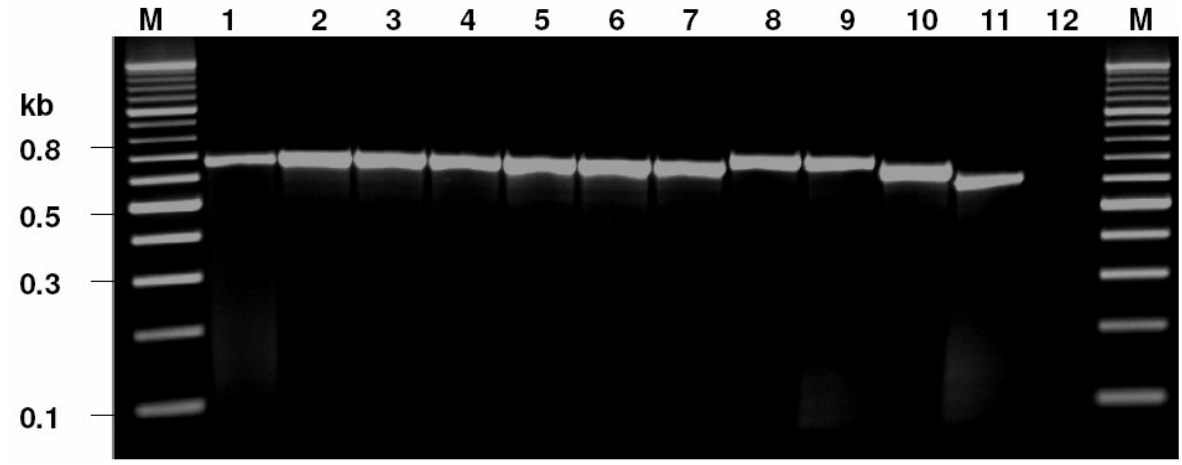

Figure 4.7: PCR products from amplification using semi-nested ribosomal RNA gene primers, 18ST nF2 and 18ST nR3, on cultured trypanosome isolates collected from KZN. In lane 1 is loaded amplified sample from isolate UPKZNBT/1/05; lane 2, UPKZNBT/4/05; lane 3, UPKZNBT/5/05; lane 4, UPKZNCT/3/05; lane 5, UPKZNCT/5/05; lane 6, UPKZNCT/11/05; lane 7, OVIKZNCT/4053/06; lane 8, OVIKZNCT/8381/06; lane 9, OVIKZNCT/8394/06; lane 10, *T. congolense* IL3000; lane 11, *T. vivax* IL2160; lane 12, water (no target DNA) and Lanes M contain the O'GeneRuler 100bp DNA ladder (Fermentas).

Those samples in which was found a 690bp product in Figure 4 were amplified with Savannah-type *T. congolense* specific primers and each of them gave a PCR product of 320bp (Figure 4.8, lanes 1-7). The samples that resulted in a 705bp product amplified with Kilifi-type *T. congolense* specific primers (Figure 4.9, lanes 8 and 9).

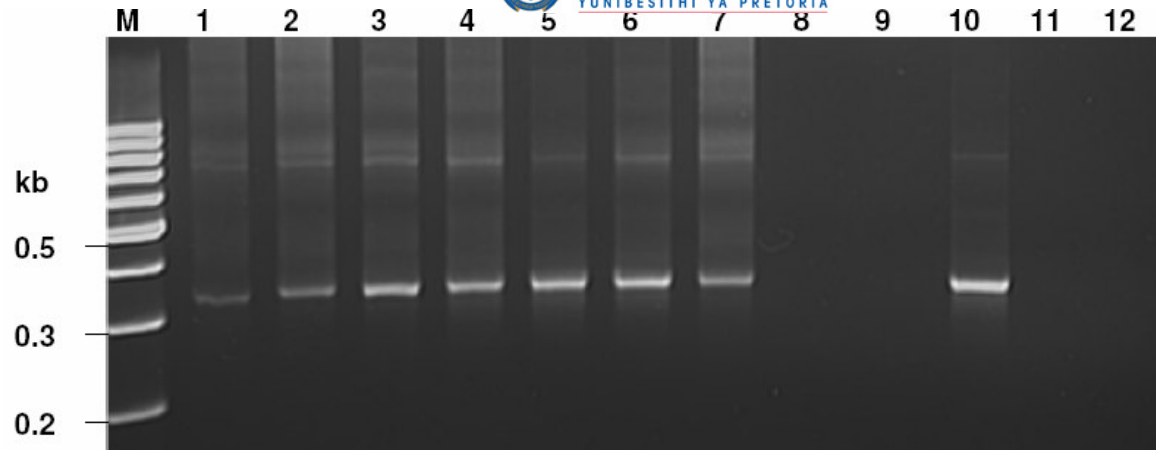

Figure 4.8: PCR products from amplification of DNA eluted from FTA elute cards on which were placed buffy coats from mice infected with each one of the nine isolates. The primers used (TCN1 and TCN2) are specific for Savannah-type *T. congolense*. In lane 1 is loaded amplified sample from a mouse infected with isolate UPKZNBT/1/05; lane 2, UPKZNBT/4/05; lane 3, UPKZNBT/5/05; lane 4, UPKZNCT/3/05; lane 5, UPKZNCT/5/05; lane 6, UPKZNCT/11/05; lane 7, OVIKZNCT/4053/06; lane 8, OVIKZNCT/8381/06; lane 9, OVIKZNCT/8394/06; lane 10, *T. congolense* IL3000; lane 11, *T. vivax* IL2160; lane 12, water (no target DNA) and lanes M contain the O'GeneRuler 100bp DNA ladder (Fermentas).

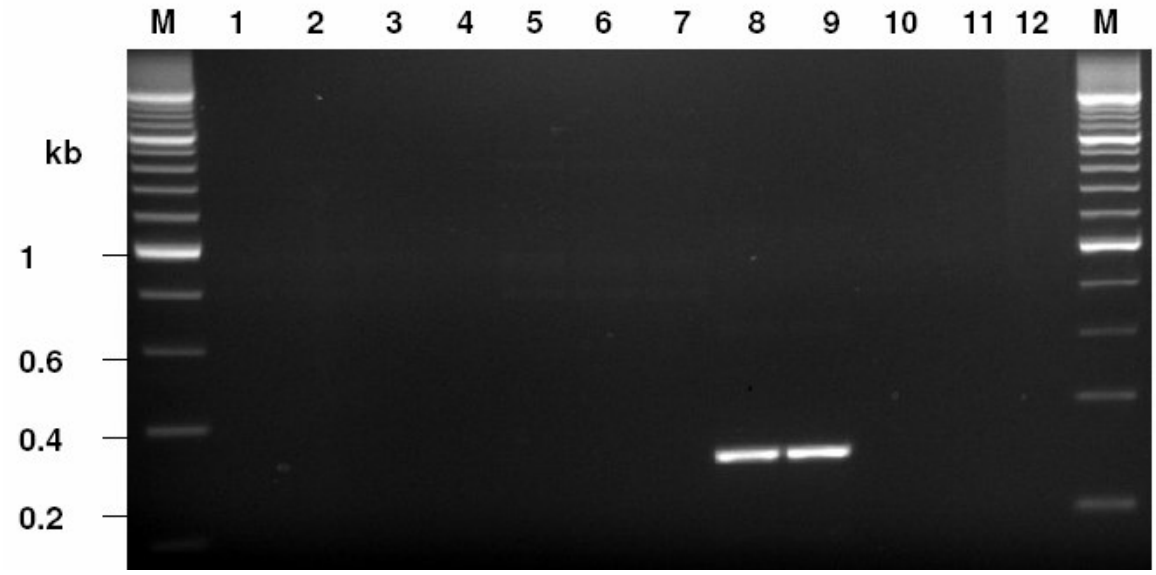

Figure 4.9: PCR products from amplification of DNA eluted from FTA elute cards on which were placed buffy coats from mice infected with each of the nine isolates. The primers used (TCK1 and TCK2) are specific for Kilifi-type *T. congolense*. In lane 1 is loaded amplified sample from a mouse infected with isolate UPKZNBT/1/05; lane 2, UPKZNBT/4/05; lane 3, UPKZNBT/5/05; lane 4, UPKZNCT/3/05; lane 5, UPKZNCT/5/05; lane 6, UPKZNCT/11/05; lane 7, OVIKZNCT/4053/06; lane 8, OVIKZNCT/8381/06; lane 9, OVIKZNCT/8394/06; lane 10, *T. congolense* IL3000; lane 11, *T. vivax* IL2160; lane 12, water (no target DNA) and lanes M the O'GeneRuler 200bp DNA ladder (Fermentas).

The rRNA gene sequences of Savannah- (accession no: U22315) and Kilifi-type (accession no: AJ009144) *T. congolense* which are amplified with 18ST nF2 and 18ST nR2 primers are shown in Figure 4.10. Restriction enzymes, *Eco*571 and *Msp*I are known to digest the

resulting PCR products to generate DNA fragments of specific sizes for each of the genotypic groups of *T. congolense*. Simplified restriction enzyme maps of each of these PCR products with approximate size fragments are shown in Figure 4.11. The maps are arranged 5' to 3' according to the sequences obtained in GenBank. In Figure 4.10, the differences between the two sequences are highlighted in blue, the recognition sequences and restriction sites for *Msp*I are highlighted in red; and the recognition sequence and restriction site for *Eco*571 is highlighted in green. *Msp*I recognizes the sequence 5' CCGG 3' and cleaves after the first C. *Eco*571 recognizes the sequence 5' CTGAAG 3' and cleaves 14-16 bases away. Insertions, deletions and conversions could have given rise to or removed restriction enzyme site and/or recognition sequences in the two sequences with respect to each other.

As indicated in Figure 4.11, for Savannah-type *T. congolense* IL3000, the recognition sequences for *Msp*I are at positions: 40, 320 and 509 and when the PCR product is digested with *Msp*I the products are: 40, 160, 189 and 280bp. The amplicon does not contain the recognition sequence for *Eco*571.

For Kilifi-type *T. congolense* the recognition sequence for *Msp*I is at position 347 and for *Eco*571 is at position 142. The expected fragments upon double digestion of the PCR product with the two restriction enzymes are: 142, 205 and 361bp.

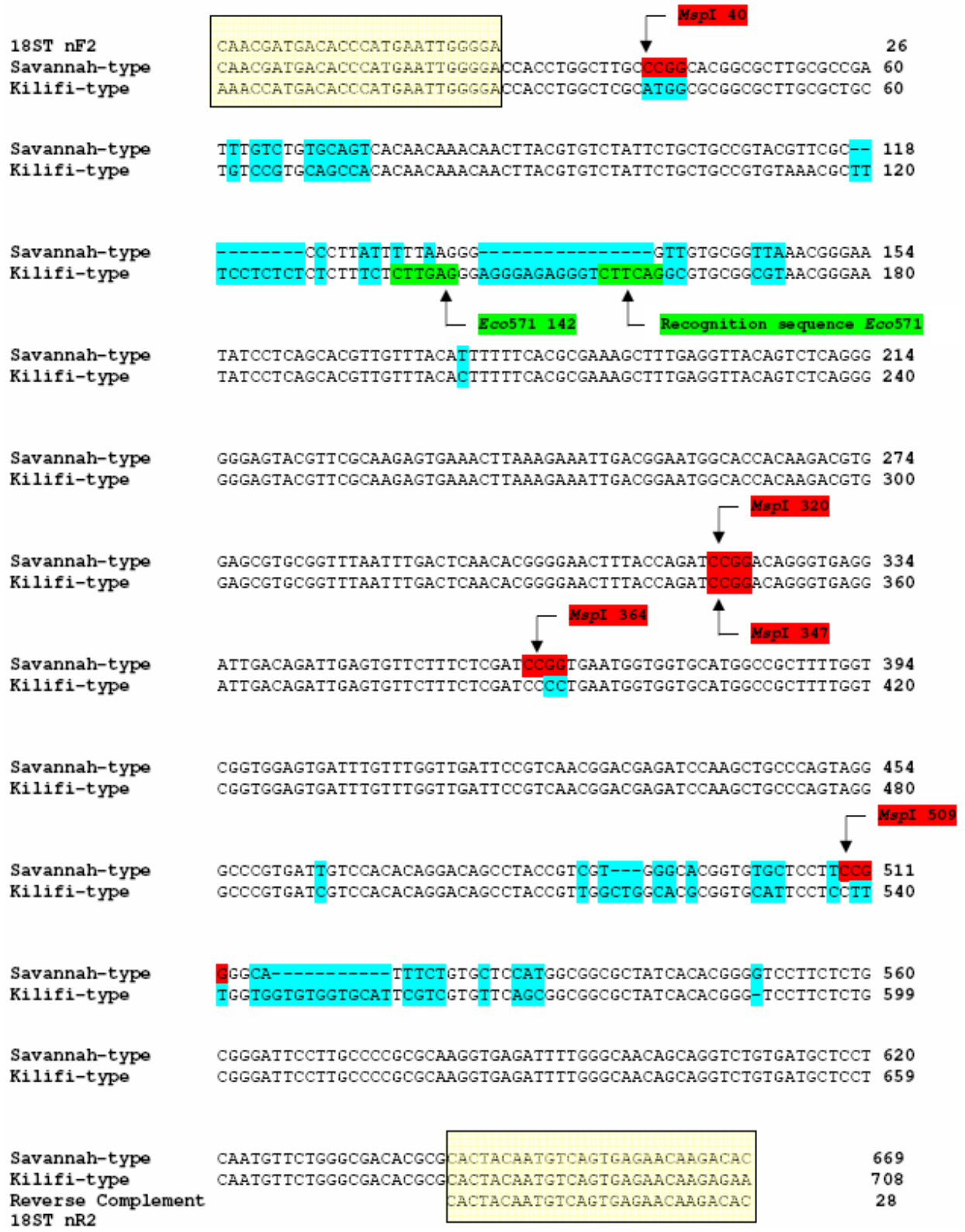

Figure 4.10: Aligned DNA sequences of the region of the rRNA gene of Savannah and Kilifi-type *T. congolense* targeted for amplification by the 18ST nF2 and 18ST nR2 primers. Position of the forward primer 18ST nF2 and the reverse complement of the reverse primer, 18ST nR2 are also shown.

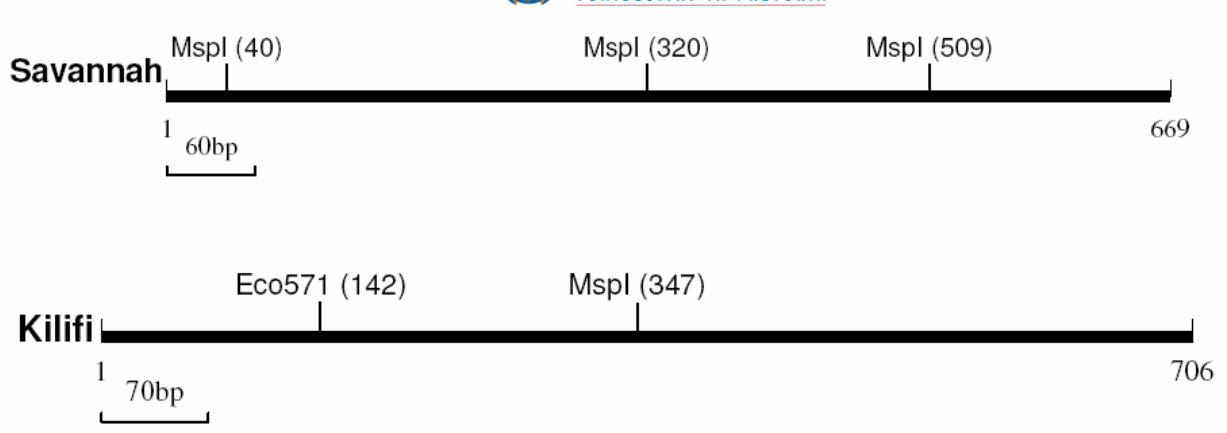

Figure 4.11: Restriction enzyme map showing the location of recognition sites for *Eco*571 and *Msp*I on the PCR product generated in PCR using rRNA gene primers on DNA of Savannah- and Kilifi-type *T. congolense.*

To clarify the identity of the parasites that resulted in a 690bp and the 705bp PCR products, the PCR products were digested with *Eco*571 and *Msp*I. The 690bp PCR product gave patterns that were similar to what was obtained for Savannah-type *T. congolense* in Figure 4.2, lane 2. These are: 160, 190 and 295bp fragments (Figure 4.12, lane 1). Digests of products in Figure 4.7, lanes 1-7 had similar profiles, hence only a representative sample is loaded in Figure 4.12, lane 1. Upon digestion of the 705bp PCR products, the fragments obtained were similar to those expected of Kilifi-type *T. congolense* i.e. 140, 205 and 360bp (Figure 4.12, lanes 2 and 3).

Therefore Savannah- and Kilifi-type *T. congolense* are present in KZN.

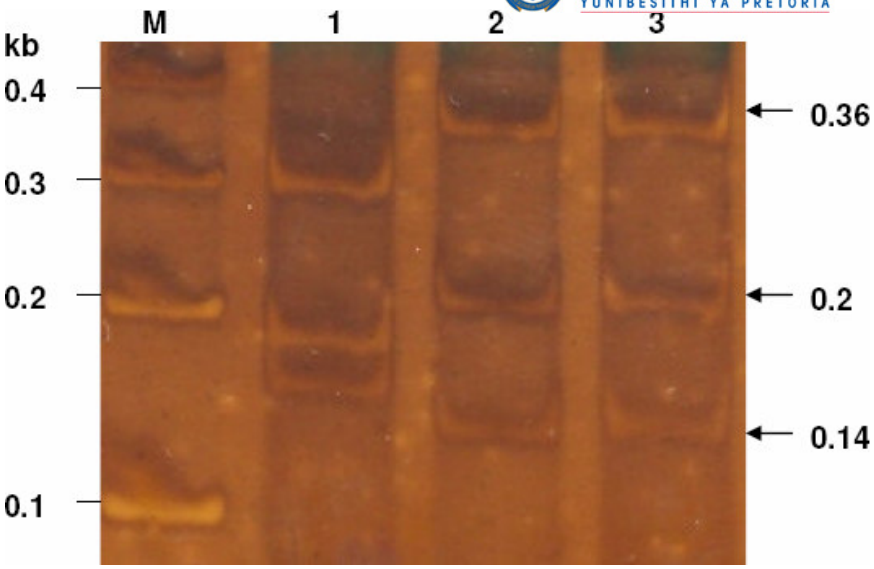

Figure 4.12: RFLP profiles generated upon double digestion (with MspI and Eco571) of PCR products obtained using 18ST nF2 and 18ST nR2 primers. In lane 1 is loaded digests the PCR product obtained upon amplification of isolate OVIKZNCT/4053/06; lane 2, OVIKZNCT/8381/06; lane 3, OVIKZNCT/8394/06 and lane M contains the O'GeneRuler 100bp DNA ladder (Fermentas).

More blood samples from cattle at different diptanks were examined for possible presence of these two genotypic groups of *T. congolense*. A summary of the results is shown in Table 4.4. At Ekuphindisweni, Mahlambinyathi and Mvutshini diptanks, all *T. congolense* infections were due to Savannah-type *T. congolense*. At Nhlajwane diptank, all four *T. congolense* positive infections that were identified were Kilifi-type. At Ndumo diptank, three out of four infections were due to Kilifi- and one was due Savannah-type *T. congolense.* At Qakweni diptank two out of the four infections were due to Kilifi- and two were Savannah-type *T. congolense.* For Boomerang commercial farm, amongst the weaners, there were 12 single infections with Kilifi, 12 single infections with Savannah and five mixed infections with Kilifi and Savannah-type *T. congolense.* With the adult cattle at Boomerang, four infections were due to Savannah- and only one was due to Kilifi-type *T. congolense*.

Both Kilifi and Savannah-type *T. congolense* are not confined to a particular geographic area KZN, they are spread throughout the districts.

Table 4.5: A summary of genotypic groups of *T. congolense* from blood samples

Location	**Kilifi**	**Savannah**	**Mixed**
Ekuphindisweni	0	2	0
Mahlambinyathi	0	15*	0
Mvutshini	0	33	0
Nhlajwane	4	0	0
Ndumo	3	1	0
Qakweni	2	2	0
Boomerang: Weaners	12	12	5
Boomerang: Adults	1	4	0

*Five of the 15 were mixed with *T. vivax*

4.6 Mixed infections in tsetse flies

The observation that the Savannah- and the Kilifi-type *T. congolense* co-exist in some areas of KZN prompted a preliminary study to determine the frequency with which the two types of *T. congolense* occur either as single or mixed infections in the tsetse vectors. The results obtained upon analysis of the second batch of flies that were mentioned in the section 4.4 are reported here. For this investigation samples were specifically collected from Boomerang commercial farm because mixed infections in cattle had been recorded there (Table 4.5). Sampling was also done at Charter's Creek and Hell's Gate Game Reserves, two game reserves that are adjacent to Boomerang. Because this study was aimed at looking specifically for the two genotypes, primers that are specific to each of them were employed in the amplifications.

A total of 126 *G. brevipalpis* were dissected and then analyzed by PCR. The proboscis and midgut of each fly was individually analyzed with each of the two primer sets. Figure 4.13a shows PCR products generated when DNA from the tsetse fly organs were amplified with primers specific for Savannah-type *T. congolense*. Figure 4.13b illustrates PCR products that were amplified with Kilifi-type *T. congolense* specific primers. In Table 4.6 is a summary of the results. Kilifi-type *T. congolense* was rarely found as a single infection in the tsetse flies examined. Only one midgut out of all the samples analyzed was positive for only Kilifi. The rest of the samples either had mixed infections or they were infected with only Savannah-type *T. congolense.*

A total of 39 out of 50 samples from Boomerang were positive, Table 4.6. Thirty-seven of the samples had only Savannah-type *T. congolense* infections, one had Kilifi-type *T. congolense,* one sample had mixed infections and 11 of the samples were negative.

At Charter's Creek there was no single Kilifi-type *T. congolense* infection, but there were 16 single Savannah-type *T. congolense* infections, 17 mixed infections and three of the samples were negative.

At Hell's Gate there was also no single Kilifi-type *T. congolense* infection, but there were 16 Savannah-type *T. congolense* single infections, 23 mixed infections and one negative sample.

Mixed infections in tsetse flies were found to occur as follows: both genotypes in the midgut and in the proboscis of a fly (Figure 4.13a and b, lanes 1 and 2); one genotype in both the midgut and proboscis, and the other genotype in either the midgut or proboscis of a fly (Figure 4.13a and b, lanes 3 and 4); and each genotype per organ (Figure 4.13a and b, lanes 5 and 6).

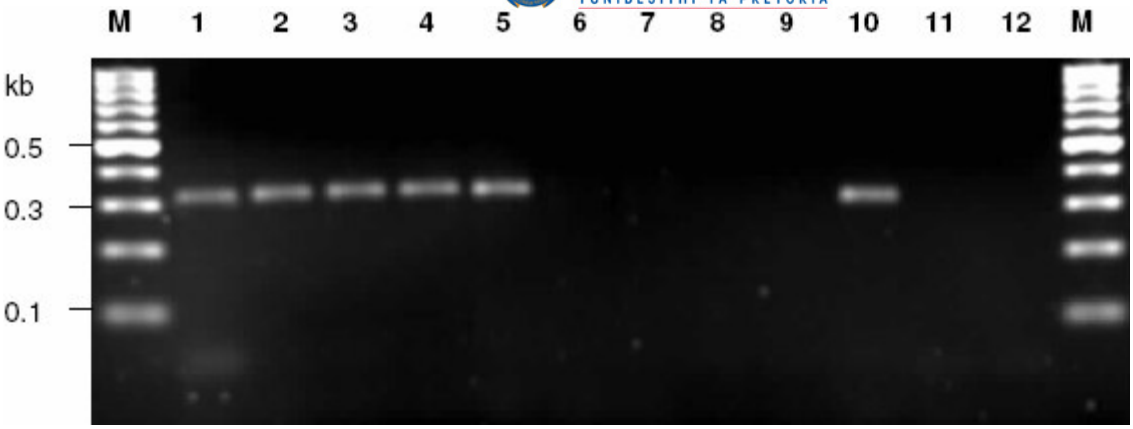

Figure 4.13a: PCR products from amplification of DNA eluted from FTA elute cards on which were blotted either the midgut or proboscis of tsetse flies. The primers used are specific for Savannah-type *T. congolense.* In lanes 1-8 are PCR products of samples from four flies. Lanes 1, 3 and 5 have products from the midguts, whilst lanes 2, 4 and 6 have products from the proboscides; lane 7, the midgut of a colony fly; lane 8, the proboscis of a colony fly; lane 9, Kilifi-type *T. congolense* DNA; lane 10, Savannah-type *T. congolense* IL3000 DNA; lane 11, *T. vivax* DNA; lane 12, water (no target DNA) and lanes M contain the O'GeneRuler 100bp DNA ladder (Fermentas).

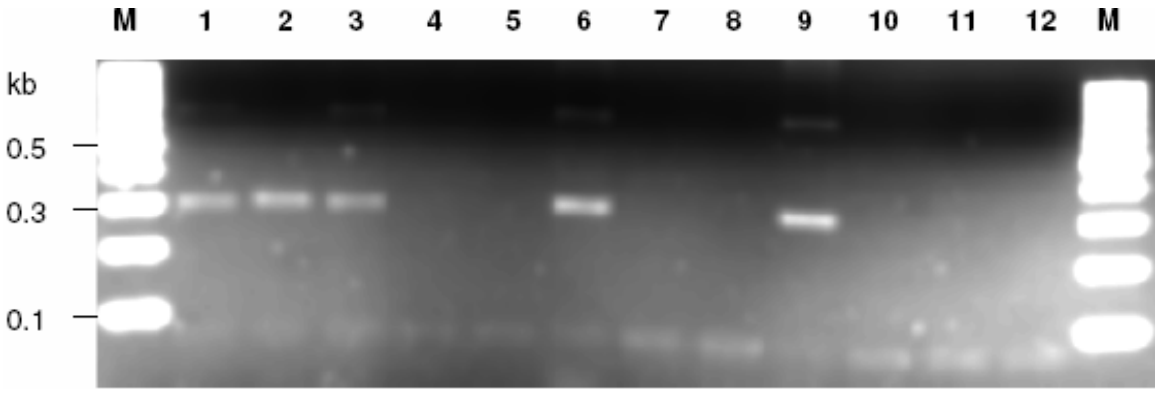

Figure 4.13b: PCR products from amplification of DNA eluted from FTA elute cards on which were blotted either the midgut or proboscis of a tsetse fly. The primers used are specific for Kilifi-type *T. congolense.* In lanes 1-6 are resulting products of samples from three flies. Lanes 1, 3, and 5 have products from the midguts, whilst lanes 2, 4 and 6 have products from the proboscides, lane 7, the midgut of a colony fly; lane 8, the proboscis of a colony fly; lane 9, Kilifi-type *T. congolense* DNA; lane 10, Savannah-type *T.congolense* IL3000 DNA, lane 11, *T. vivax* DNA; lane 12, water (no target DNA) and lanes M contain the O'GeneRuler 100bp DNA ladder (Fermentas).

The overall infection rate of tsetse flies collected from the abovementioned locations was 89%. Mixed infections varied from 2% to 57%, the highest being at Hell's Gate.

Table 4.6: A summary of PCR results that were obtained upon analyzing samples from *G. brevipalpis.* The samples were amplified with primers that are specific for Savannah- or Kilifi-type *T. congolense*.

	Boomerang	**Charter's Creek**	**Hell's Gate**
Single Kilifi	1	0	0
Single Savannah	37	16	16
Mixed	1	17	23
Negative	11	3	1
Total	50	36	40
Infection rate	78%	91.6%	97.5%

4.7 Genetic diversity of Savannah-type *T. congolense* in KZN

The degree to which genomes are similar to each other is reflected in the number of bands they have in common when they are subjected to random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland, 1990).

Two primers of arbitrary nucleotide sequence were used to evaluate the variation among the Savannah-type *T. congolense* isolates collected in KZN. For RAPD analysis, it is imperative to use DNA of good quality although some level of degradation can be tolerated (Dias Neto *et al.,* 1997). The extracted DNAs of the isolates that had to be compared were firstly resolved by electrophoresis in an agarose gel to assess their integrity. Figure 4.14 shows that they are of comparable quality, although the DNA in lane 3 shows some degradation.

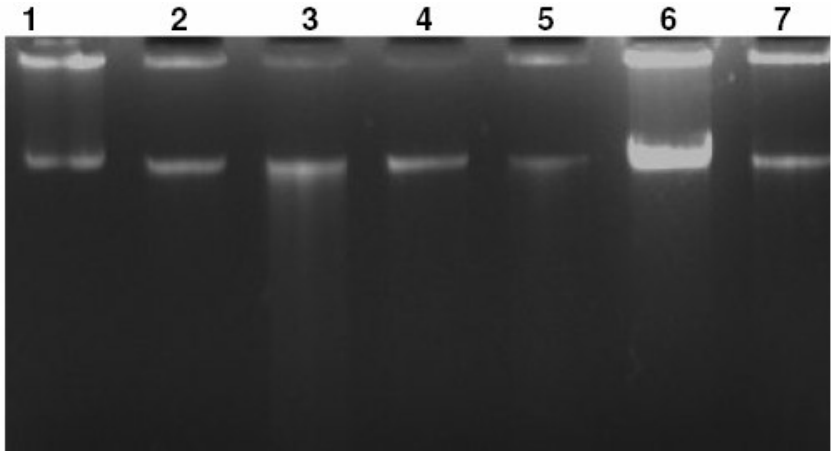

Figure 4.14: Integrity or intactness of isolated DNAs to be used in RAPD analysis. The DNAs in lanes 1, 2, 3, 4, 5, 6 and 7 were isolated from OVI1, UPKZNBT/2/06, UPKZNBT/4/06, UPKZNCT/5/05, OVIKZNCT/4053/06, OVIKZNCT/7098/06, and IL3000 respectively.

The quality of *Taq* polymerases used in amplifications can influence the outcome of a PCR in various ways. It is known that *Taq* polymerases that are available on the market differ in their respective fidelities, sensitivities and specificities (Mendelman *et al.,* 1989), properties which can influence their ability to amplify a target. *Taq* polymerases from two different sources were compared for their ability to randomly amplify a set of DNA samples. With the one *Taq* polymerase, only a few non-informative bands were obtained (Figure 4.15; lanes 1, 3, 5 and 7). When the same samples were subjected to amplification under identical conditions, but using the other *Taq* polymerase, more bands ranging in size from 0.3kb to 3.5kb were observed (Figure 4.15; lanes 2, 4, 6 and 8). All further amplifications were performed using the second *Taq* polymerase.

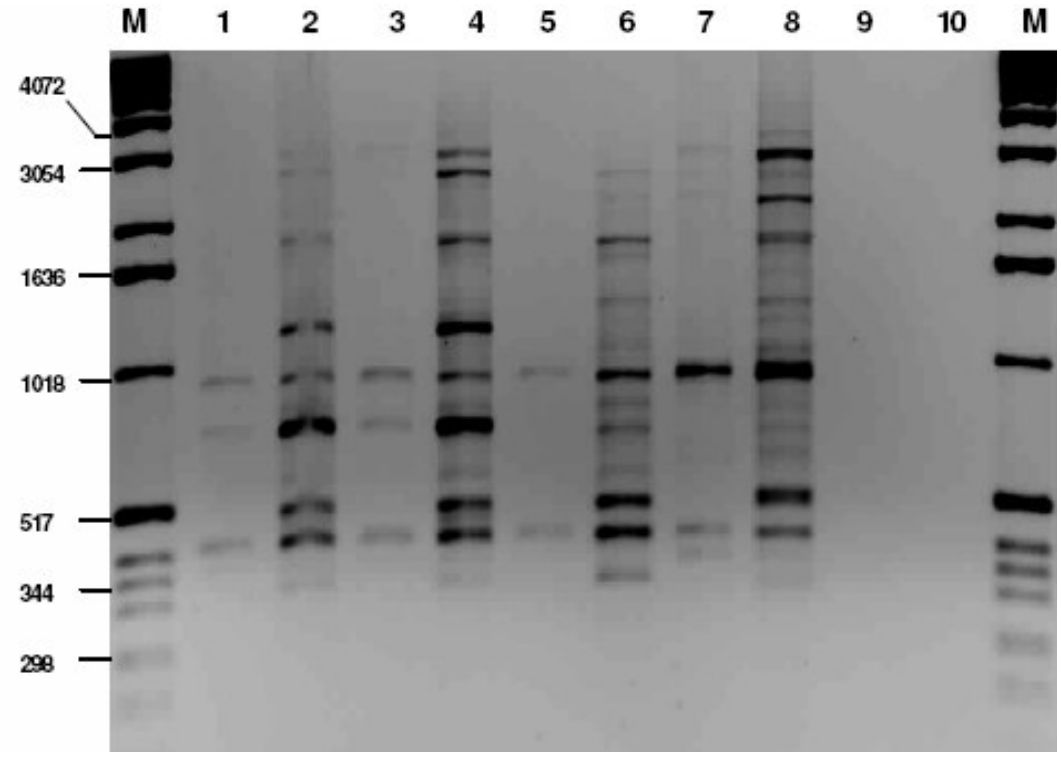

Figure 4.15: Assessing the quality of two different *Taq* polymerases. Products obtained with *Taq* polymerase 1 are loaded in lanes 1, 3, 5, 7 and 9; and products obtained with *Taq* polymerase 2 are loaded in lanes 2, 4, 6, 8 and 10. Products obtained from identical targets were loaded in lanes 1 and 2; 3 and 4; 5 and 6; 7 and 8; 9 and 10 had water and lanes M contain molecular Marker X (Roche).

Two different arbitrary primers (ILO509 and ILO524) were tested for their ability to distinguish among the three most important species of trypanosomes that affect livestock (*T. brucei*, *T. congolense* and *T. vivax*), two genotypic groups of *T. congolense* (Savannah and Kilifi), as well as *T. equiperdum* and *T. brucei.* As is evident in Figure 4.16a and b, both primers can distinguish *T. brucei*, *T. congolense* and *T. vivax* from one another (Figure 4.16a and b: lanes 2, 3 and 5). The primers can also distinguish *T. equiperdum* from *T. brucei* (Figure 4.16a and b, lanes 1 and 2) as well as the Savannah- from the Kilifi-type *T. congolense* (Figure 4.16a and b, lanes 3 and 4) as reflected by the banding patterns.

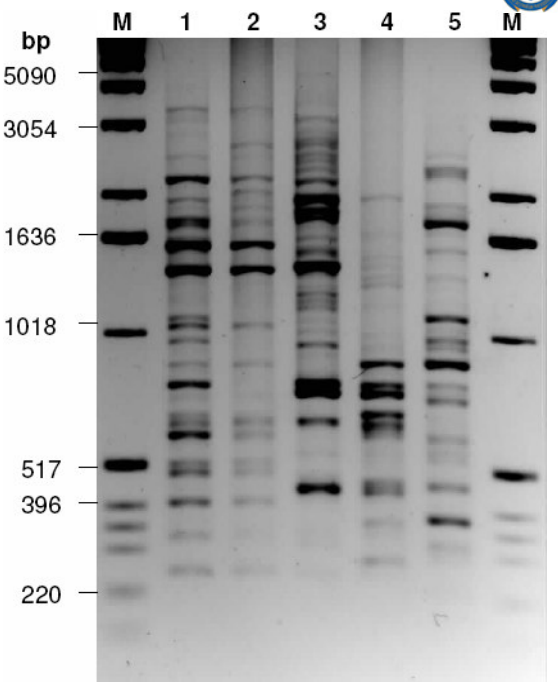

Figure 4.16a: RAPD profiles generated using the ILO524 primer. In lane 1 was loaded amplified fragments of *T. equiperdum*; lane 2, *T. brucei;* lane 3, Savannah-type *T. congolense*; lane 4, Kilifi-type *T. congolense*; lane 5, *T. vivax* and lanes M, Marker X (Roche).

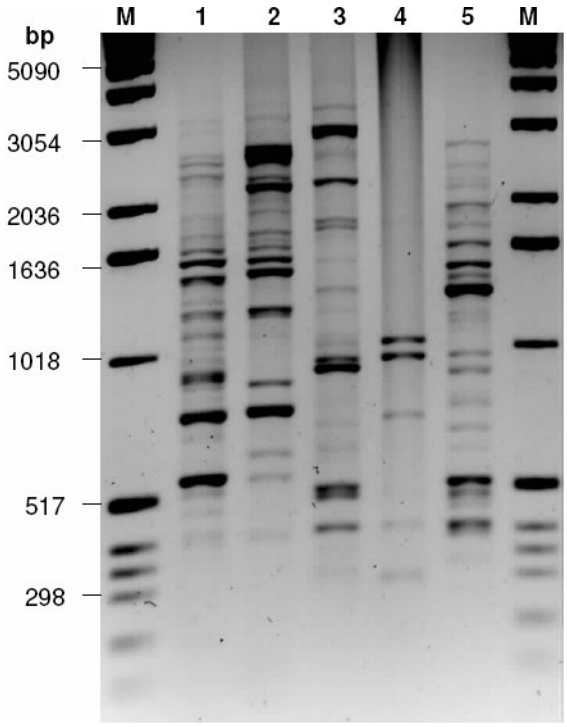

Figure 4.16b: RAPD profiles generated using the ILO509 primer. In lane 1 was loaded amplified fragments of *T. equiperdum*; lane 2, *T. brucei;* lane 3, Savannah-type *T. congolense*; lane 4, Kilifi-type *T. congolense*; lane 5, *T. vivax* and lanes M, Marker X (Roche).

Having confirmed that each of the two arbitrary primers can distinguish trypanosomes from species to genotypic level, the primers were then used to investigate genome variation, if any, among the nine isolates of Savannah-type *T. congolense*, five of which were from buffaloes and four from cattle. The DNAs used were all intact (no degradation), of comparable qualities (their 260/280 ratios were approximately 1.8, and their 260/230 ratios were between 1.8 and 2.2) and the same quantities (50ng) were used in the amplifications. DNA absorbs UV light at 260nm, proteins at 280nm and other organic compounds at 230nm. The 260/280 and 260/230 ratios give an indication of the purity of nucleic acids with respect to protein contamination and other organic compounds respectively. A 260/280 ratio of between 1.8 and 2.0, and a 260/230 ratio of 1.8 and 2.2 is accepted as sufficient for DNA.

All the isolates analyzed had been concluded to be Savannah-type *T. congolense* based on the size of their PCR product obtained using ribosomal RNA gene primers (seven illustrated in Figure 4.7), their RFLP profiles (one illustrated in Figure 4.12) and amplification with Savannah-type *T. congolense* specific primers (seven illustrated in Figure 4.8).

In the RAPD analyses, the number and size of amplified fragments varied depending on the primer used. The amplification pattern generated with the ILO524 primer comprised fragments of sizes ranging from 0.25kb to 4kb (Figure 4.17). Overall, no significant differences were observed in banding patterns among the isolates with the ILO524 primer.

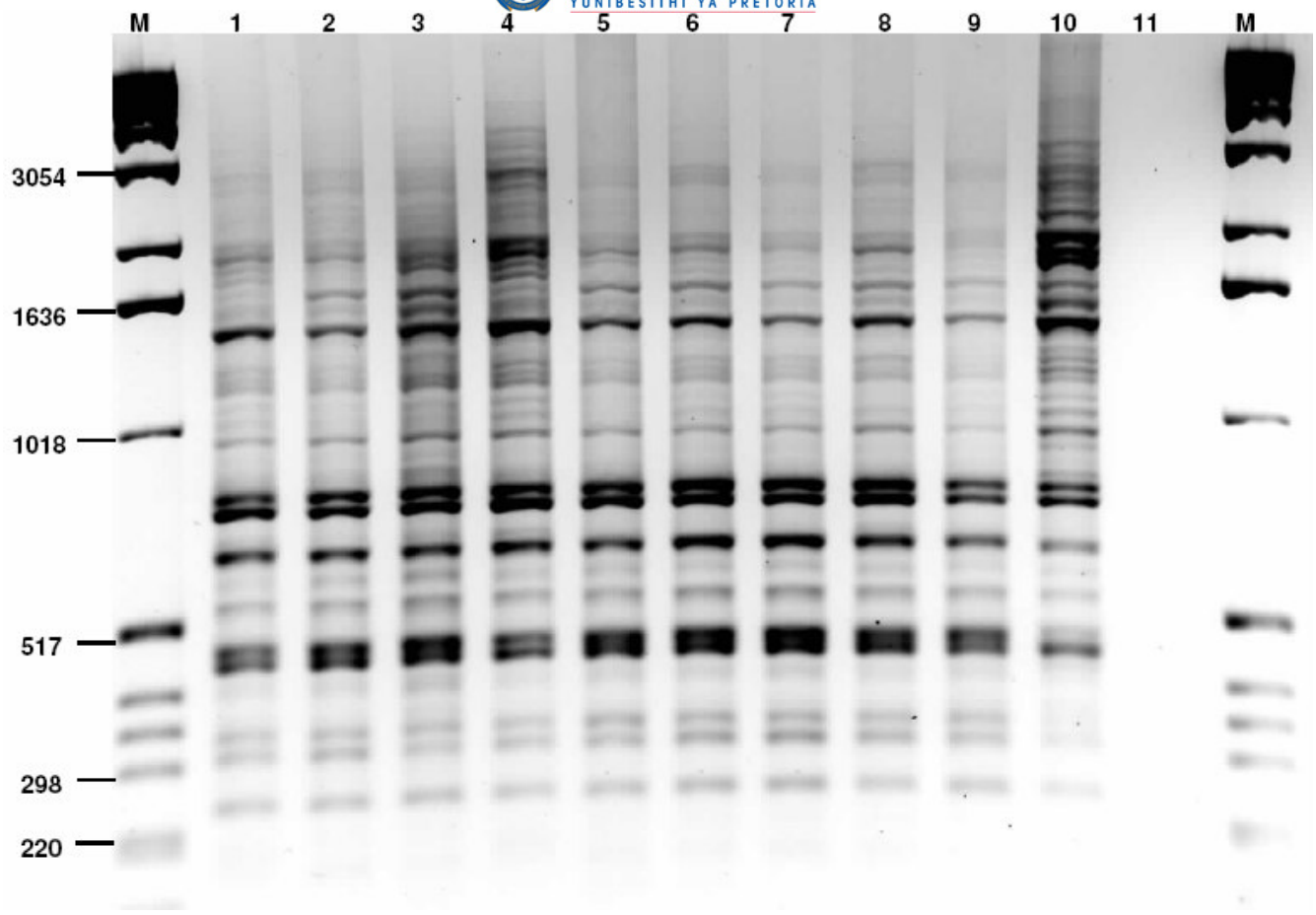

Figure 4.17: RAPD profiles generated using ILO524 primer. In lane 1 are RAPD products of DNA purified from isolate UPKZNBT/1/06; lane2, UPKZNBT/2/06; lane 3, UPKZNBT/3/06; lane 4, UPKZNBT/4/06; lane 5, UPKZNBT/5/06; lane 6, UPKZNCT/3/05; lane 7, UPKZNCT/5/05; lane 8, UPKZNCT/11/05; lane 9, OVIKZNCT/4053/06; lane 10, IL3000; lane 11, water (no target) and lanes M, Marker X (Roche).

The amplification pattern generated with the ILO509 primer comprised eight to 22 fragments with sizes ranging from 0.3kb to 4kb (Figure 4.18).

The ε-bands in Figure 4.18 are two of the six bands (320bp and 800bp) that are common to all the isolates as well as the IL3000 control. The δ-bands (520bp and 1000bp) appeared to be unique to IL3000 (Figure 4.18, lane 10). The α-bands (2750bp) are only present in the Savannah-type *T. congolense* isolates from KZN (Figure 4.18, lanes 1-9). The β-bands (1300bp) are present only in two of the nine isolates; UPKZNBT/4/06 and UPKZNCT/11/05 (lanes 4 and 8), whereas the ζ-bands (475bp) are present in three isolates; UPKZNBT/4/06 and UPKZNCT/11/05, two of which share the β-bands (lanes 1, 4 and 8). The γ-bands (750bp and 1200bp) are present in all the isolates from buffaloes and one from cattle (OVIKZNCT/11/05) (lanes 1-5 and 8). The γ_1-band is also shared with the IL3000 clone. In summary, there appears to be at least five different RAPD band groups of Savannah-type *T.*

congolense in KZN as revealed by ILO509 primer. The groups are as follows: isolate UPKZNBT/1/06 belong to the first RAPD Group (RG); isolates UPKZNBT/2/06, UPKZNBT/3/06 and UPKZNBT/5/06 belong to the second RG; isolate UPKZNBT/4/06 belong to the third RG; isolates UPKZNCT/3/05, UPKZNCT/5/05 and OVIKZN/4053/06 belong to the fourth RG and isolate UPKZNCT/11/05 belong to the fifth group.

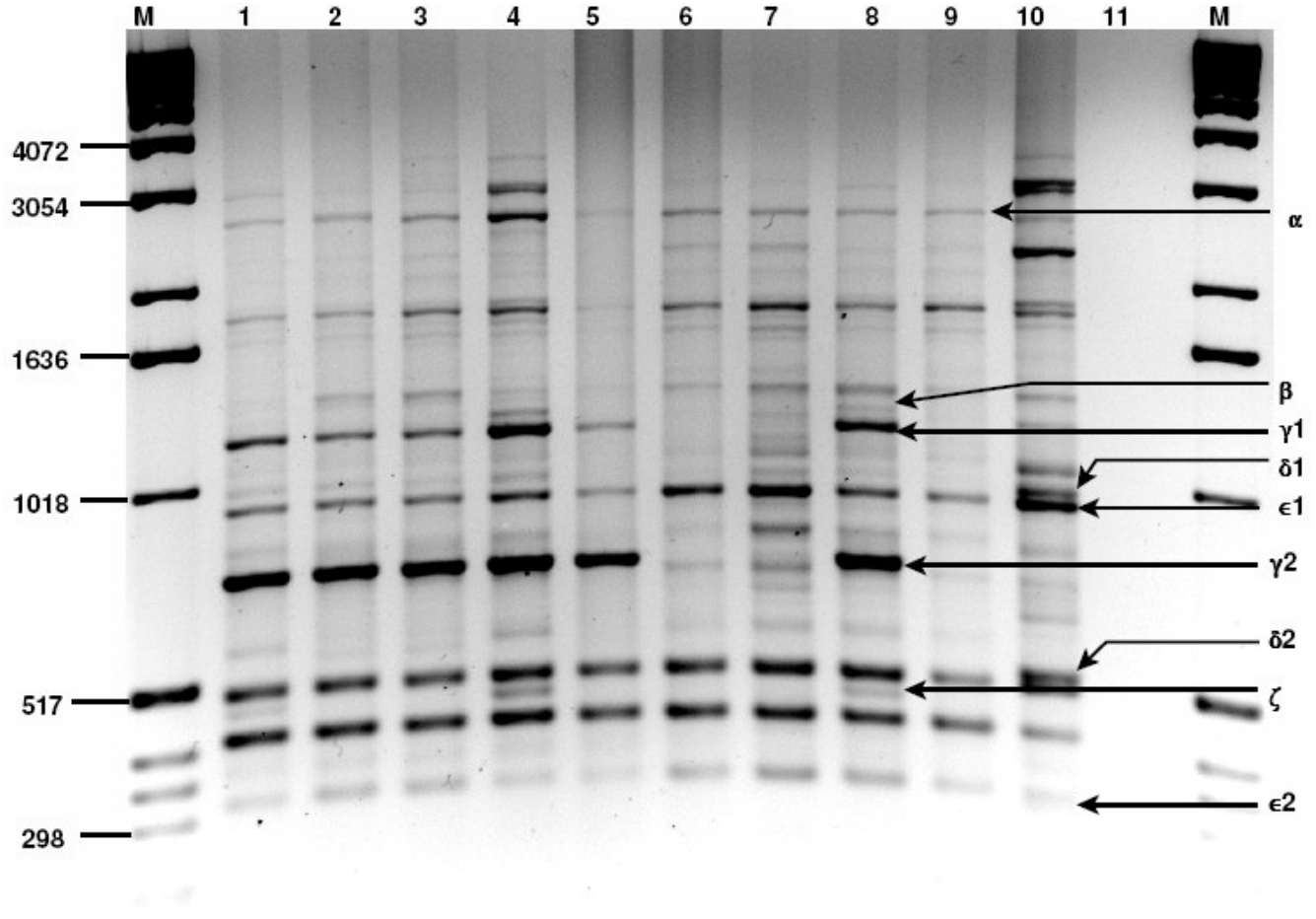

Figure 4.18: RAPD profiles generated using ILO509 primer. In lane 1 are RAPD products of DNA purified from isolate UPKZNBT/1/06; lane2, UPKZNBT/2/06; lane 3, UPKZNBT/3/06; lane 4, UPKZNBT/4/06; lane 5, UPKZNBT/5/06; lane 6, UPKZNCT/3/05; lane 7, UPKZNCT/5/05; lane 8, UPKZNCT/11/05; lane 9, OVIKZNCT/4053/06; lane 10, IL3000; lane 11, water (no target) and lanes M, Marker X (Roche).

CHAPTER 5

5. DISCUSSION AND CONCLUSION

Over the years, several DNA sequences of trypanosomes have been used for the design of sensitive and specific PCRs for the detection of these parasites in their vectors and in the blood of their vertebrate hosts. This has made it possible to detect and characterize these parasites more rapidly and with greater precision.

5.1.1 Specificity of ribosomal RNA gene primers

In investigating whether the 18ST PCR could be used in characterizing trypanosomes from KZN, the reaction was initially performed on the DNA of the three livestock-infective trypanosomes in a semi-nested PCR (Figure 4.1). The semi-nested approach improves the sensitivity of the detection, and results in higher quantity of product to enable digestion with restriction enzymes. This is necessary in situations where the parasitaemia is low and below the detection limit of ordinary PCR.

The difference in the size of the PCR product may not always be sufficient for the differentiation of species, especially when amplicon sizes differ by a few base pairs. For example, the expected size of the PCR product from the semi-nested amplification of Savannah-type *T. congolense* rRNA gene is 671bp, whereas the product for *T. brucei* is 660bp, as predicted from the nucleotide sequences of these loci. It is not always possible to visualize the 11bp difference in an ordinary agarose gel. However, upon digestion of the PCR products with selected restriction enzymes, the differences become more apparent. The coupling of this PCR with RFLP analysis thus provided a tool to differentiate trypanosome species whose PCR products may be similar in size but different in nucleotide sequence composition. The amplicons were digested with *Msp*I and *Eco*571 since they possess recognition sequences of one or both of the enzymes and the size of the fragments following restriction are different for each species of trypanosome as shown in Figures 4.2, 4.10 and 4.11.

The disadvantage of using this approach is that the amplicon has to be digested with restriction enzymes in order to reveal the polymorphism among species. This is tedious, time consuming and expensive. Adams *et al*. (2006) have attempted to overcome this limitation by designing generic primers that anneal to the ITS-1 region of rRNA gene. Unlike the 18S

rRNA gene PCR that was used in this study, their test does not involve a nested amplification step and shows distinct differences in the size of the PCR products from different trypanosome species. By using this approach, it would be possible to lower the cost of PCRs, for example, by performing only one round of amplification and not using restriction enzymes. The approach was not taken in this study because the findings were published while this project was in progress.

5.1.2 Sensitivity and specificity of species-specific primers

Ribosomal RNA gene primers and the species-specific primers were compared for their ability to amplify trypanosome DNA in test samples. Figure 4.5 demonstrates how the ribosomal primers failed to reveal Savannah-type *T. congolense* in one of the five positive samples, which were amplified in Figure 4.6 with Savannah-type *T. congolense* specific primers. The recorded sensitivity of species-specific primers and those targeting the 18S rRNA gene (using the nested approach) is a minimum one trypanosome genome per 50µl and 25µl reaction respectively (Masiga, 1992; Geysen *et al.,* 2003). So, the former primers are two times more sensitive than the latter. This further indicates that species-specific primers are more sensitive than the rRNA gene primers. This can be attributed to the fact that the copy number of the rDNA locus in trypanosomes ranges from 100-200 copies (Desquesnes *et al.,* 2001). The copy number of the satellite DNA targeted for *Nannomonas*-specific amplification has not been recorded, but is thought to be over 1000 in a single trypanosome genome (trypanosomes are di-allelic). So the amount of target is higher for species-specific primers, thus less parasites are required for DNA amplification and the amount of PCR product obtained is higher.

In some instances primers can amplify more than one tandem repeat thus giving rise to several specific bands (Figure 4.5, lane 11 and Figure 4.6, lane 9). A sample was always regarded positive when an amplicon, whose size is a multiple of a single unit was observed.

In order to screen samples for the presence of different species of trypanosomes, the 18ST primers can be used as they will anneal to conserved regions in the 18S rRNA genes of most trypanosomatids including *T. theileri*. In order to determine the frequency with which a particular species of trypanosome occurs, species-specific primers would have to be used. To determine the different species of trypanosomes in cattle, wild animals and tsetse flies, 18S rRNA gene primers were used. They gave an idea as to the different species of trypanosomes present in KZN.

5.1.3 Species of trypanosomes in KZN

This study used molecular methods to confirm the presence of *T. congolense* and *T. vivax* in KZN. The infection rate in cattle at the 14 diptanks and one commercial farm ranged from 5.2% to 91%. Cattle sampled at Mvutshini diptank had the highest infection rate, i.e., 91%. This is the same diptank from where Van den Bossche *et al.* (2006b) sampled cattle in 2006 and found an infection rate of 60.5% as determined by PCR. The observed difference in infection rates of 2006 by Van den Bossche can be attributed to the fact that Van den Bossche performed random sampling whereas the sampling in this study was biased. Infection rates of 67% and 12.5% were also observed in the weaners and adult cattle respectively at Boomerang. The difference in infection of the young cattle compared to the adult cattle could be due to the fact that the adult herd was previously treated, whereas the weaners had never been treated for trypanosomosis.

It is noteworthy that sampling of cattle was biased in that the samples were taken from emaciated and sick-looking animals. So, other species of trypanosomes that do not give rise to prominent symptoms could have been missed if they were present. On the other hand, not all emaciated cattle were infected by trypanosomes detectable by the methods used, and not all emaciated animals in KZN are infected with trypanosomes. This study aimed to determine the species of trypanosomes that were present in KZN, and the obvious (first) place that was looked at was tsetse flies, cattle that were displaying typical symptoms of *nagana,* and only two of the known reservoirs of trypanosomes. As such, our sampling strategy was uncontrolled and lacked statistical significance.

Wild animals are generally trypanotolerant, and as a result, they can harbour pathogenic trypanosomes for long periods of time, whilst acting as a source of blood meal, and hence trypanosomes for tsetse flies. Five out of 141 buffaloes from Hluhluwe-Umfolozi Game Reserve were positive for *T. congolense,* whilst the six rhinos which were being relocated to Pongola Game Reserve were free of detectable trypanosomes at the time of sampling.

In KZN there are many man-made tsetse habitats such as pine trees, timber, banana and sugar cane plantations (Kappmeier and Nevill, 1999). The presence of suitable habitats and vertebrate hosts ensures the persistence of tsetse flies in an environment. The level of threat that they pose to livestock and humans can be assessed by identifying the species of

trypanosome that they carry. Both *G. brevipalpis* and *G. austeni* were caught in the traps that were set, and the former was the predominant species.

From the first batch of flies (140), proboscides and midguts were examined by microscopy for motile trypanosomes. In this way, the overall infection rate in the flies was found to be 14% (20/140). Morlais *et al.* (1998) had described a similar proportion in naturally infected flies in Cameroon. The 20 positive organs were injected into mice, one midgut and 19 proboscides. The reason one midgut was injected into a mouse was that it was evident that the tsetse had just had a blood meal, and there was a chance that the bloodstream form trypanosomes may not have converted to procyclic forms. Proboscides were injected into mice because the infection there represents the potential challenge to livestock.

The mice did not develop detectable parasitaemia; nevertheless trypanosome DNA was amplified in the buffy coats of all the 20 mice when primers specific for Savannah-type *T. congolense* were used in PCR. This further demonstrated the superiority of PCR over microscopy in identifying trypanosomes.

The second batch of flies (126) was not examined for presence of trypanosomes by microscopy, but parasite DNA was amplified directly from the samples using primers that are specific for Savannah- or Kilifi-type *T. congolense*. There, the overall infection rate was 89% (Table 4.6).

Trypanosoma brucei was named after Dr David Bruce following his discovery of this parasite in the blood of cattle at Ubombo in KZN in 1895. The location where he first saw the trypanosomes is a few kilometres north of the current study area. However, that species of trypanosome was not detected in any of the samples that were analyzed. This can either mean that there is no *T. brucei* in the parts of KZN from where samples were collected, or that parasitaemia in the samples were at levels that are below the detection limit of the technologies used in the current study.

Trypanosoma congolense was by far the most abundant species of trypanosomes in KZN, accounting for almost three quarters (73%) of all infections. This species was widespread in the study area, being present at six diptanks (Ekuphindisweni, Mahlambinyathi, Mvutshini, Ndumo, Nhlajwane and Qakweni) that surround the Hluhluwe-Umfolozi Game Reserve. The number of *T. vivax* infections was small. This species was nonetheless not confined to a particular area, as it was observed at three diptanks that lie north of the Hluhluwe-Umfolozi

Game Reserve (Ekuphindisweni, Nhlwati and Qakweni) (Figure 3.1). Mixed infections of *T. congolense* and *T. vivax* were found in samples collected from cattle at Mahlabinyathi diptank.

5.1.4 Genotypic groups of *T. congolense* in KZN

PCR with primers that target the ribosomal RNA gene, a range of species-specific primers and RFLP analysis established the presence and co-existence of at least 2 genotypic groups of *T. congolense* (Kilifi- and Savannah-type) in KZN (Figures 4.5 and 4.6).

In the study, Savannah-type *T. congolense* was found at Boomerang commercial farm as well as at five of the 14 diptanks (Ekuphindisweni, Mahlambinyathi, Mvutshini, Ndumo and Qakweni). Overall, this genotype was present in 60% of all *T. congolense* single infections in cattle (Table 4.5). In wild animals that were sampled, only Savannah-type *T. congolense* was detected. In tsetse flies, Savannah-type was detected in 89 single infections in a total of 131 infections (Table 4.5 and 4.6). This genotype has adapted to the various environments in Africa and its one of the subspecies of *T. congolense* that is commonly found in livestock (Njiru *et al.,* 2004).

Kilifi-type *T. congolense* was found in samples from Boomerang commercial farm and at three of the 14 diptanks (Ndumo, Nhlajwane and Qakweni). This genotype was not detected in the samples from wild animals. It was also rarely found as a single infection in tsetse flies, occurring in only one out of 126 flies. It was rather unexpected to find this genotype in South Africa because it was previously thought to be confined to the coastal area of Kenya. Then it was later observed in the interior of Kenya, Uganda and later in the Morogoro district in Tanzania (Majiwa and Otieno, 1990; Nyeko *et al*., 1990, Mugittu *et al.,* 2001). Evidence has now been provided for presence of Kilifi-type *T. congolense* in South Africa.

Dissection and microscopy have been widely used for the identification of trypanosomes harboured by tsetse flies. However, mixed *Nannomonas* infections cannot be identified with this method. The use of Savannah-type and Kilifi-type *T. congolense* specific primers in PCR has allowed for the identification of mixed infections in flies, that would have otherwise been classified as just *T. congolense* due to their similar morphology.

A total of 46 mixed infections of Savannah- and Kilifi-type *T. congolense* were detected in all the samples examined. Five of those were in the weaners that were sampled at Boomerang

commercial farm, and the other 41 were in tsetse flies. The simplest route through which a tsetse fly can acquire a mixed infection is by its feeding on a host that is infected with different species or sub-species (concurrent infection). Another way is when a fly successively feeds on two or more infected hosts each of which is infected with a different species of trypanosome (sequential infection) (Morlais *et al.,* 1998). Subsequent blood meals by that particular tsetse vector could result in the transmission of a mixed infection. The fact that Kilifi-type *T. congolense* was mostly found to be mixed with Savannah-type *T. congolense* in tsetse flies, and rarely individually may lead to speculations that perhaps the former genotype may have recombined with the latter, and what is interpreted as mixed infections may be hybrids of the two genotypes. Evidence has been provided for genetic exchange between *T. brucei* stocks (Gibson and Stevens, 1999; Tait *et al*., 2007), and recently the mating between Savannah-type *T. congolense* strains were shown to be possible (Tait, unpublished). The possibility of genetic exchange between Savannah- and Kilifi-type *T. congolense* has not yet been shown, and cannot be ruled out. Unfortunately the scope of this study did not extend that far and the phenomenon will be a subject for future studies.

5.1.5 Genome variation of Savannah-type *T. congolense* in KZN

When chromosome-sized DNA molecules were comparatively separated using electrophoretic techniques, differences in molecular karyotypes of trypanosomes that belong to *T. congolense* species were found. It was observed that chromosome profiles of clones that belonged to different antigenic repertoires differed in size and number of bands (Masake *et al.,* 1988). Further studies showed that trypanosome clones that belong to the same antigenic repertoire display similar overall chromosome profiles, with regard to the number and size of the bands (Majiwa *et al.,* 1993). Another study observed that trypanosomes of the same genetic background have similar RAPD banding patterns (Majiwa, Unpublished). The primer which was used in the latter finding (ILO509) was included in RAPD PCR in this study. The other primer which was used, ILO524, previously distinguished between two distinct antigenic repertoires (Majiwa *et al*., 1993).

Both primers that were used in RAPD analysis could differentiate the three livestock-infective trypanosomes, genotypic groups of *T. congolense* (Savannah and Kilifi) and the closely related *T. brucei* and *T. equiperdum* (OVI1). The primers were then used to analyze the genomes of nine isolates of Savannah-type *T. congolense* from KZN. Only Savannah-type *T. congolense* isolates were selected for analysis because this genotype is predominant in KZN

and a study by Bengaly *et al.* (2002) showed that this genotypic group is more pathogenic, and consequently causes more economic losses than Kilifi-type.

The DNA which was subjected to RAPD PCR was purified from trypanosome isolates that were grown in culture. Care was taken so as to remove all host cells. First the culture conditions are unsuitable for growth of host cells. Secondly, the cultures were maintained for at least 21 days, by which time all host cells would have died out. Additionally, the cultures were serially diluted during each passage to ensure that none of the host cells were carried over to the next culture. Filtration or removal of host cells is pivotal because amplification of host DNA will interfere with the analysis.

RAPD profiles generated with the ILO524 primer appear to be similar (Figure 4.17) between 220bp to 1018bp, but vary significantly above 1500bp. The differences are made more apparent when another primer, ILO509, is used (Figure 4.18). A prominent band of 750bp common to all the buffalo isolates and present also in cattle isolates in lane 8 indicates that the parasites of the same genotype occur in both wildlife and domestic livestock. In fact, the RAPD pattern of trypanosomes in lanes 3 and 8 are indistinguishable. The RAPD profiles obtained with this primer can be clustered into five groups as follows: group 1, UPKZNBT/1/06; group 2, UPKZNBT/2/06, UPKZNBT/3/06 and UPKZNBT/5/06; group 3, UPKZNBT/4/06; group 4, UPKZNCT/3/05, UPKZNCT/5/05 and OVIKZN/4053/06 and group 5, UPKZNCT/11/05. The differences in results with the two primers can be attributed to the fact that the ILO524 primer binds to loci that do not vary in the genome of these parasites. This primer was deemed less informative for investigating genome variation among KZN isolates of *T. congolense*. The ILO509 primer is more informative in this respect because it has the ability to reveal several polymorphic bands. RAPD patterns were reproducible, but the patterns changed when the samples were analyzed after 11 months of storage at -20ºC, perhaps due to the fact that DNA storage was not optimal.

The RAPD PCRs were performed using DNA purified from isolates that were grown in mice and then in culture. The RAPD patterns appear to be simple indicating that the parasites could be clonal. It is possible that the trypanosomes could have undergone selection on their ability to grow in mice and subsequently in culture. One other consideration is that the number of isolates analyzed was small, and the isolates were collected from only three different locations. Two of the locations are in close proximity (0.5km apart) and it would be expected that the same trypanosomes can be found in both locations. Furthermore, eight of the nine

isolates were collected from the aforementioned two locations, and only one of the isolates was collected at a farm further away. RAPD analysis in this project was performed upon amplification of the nine isolates with only two arbitrary primers; more comprehensive information can be obtained by using additional random primers.

Existence of two Savannah-type *T. congolense* clones (IL1180 and IL3200) that exhibits different responses to diminazene aceturate were described by Burudi *et al.* (1994). The two clones belong to different antigenic repertoires and co-incidentally exhibit different phenotypes in terms of their response to diminazene aceturate. The former is sensitive to diminazine treatment whilst the latter is relatively more tolerant to the drug. The next step of this study would be to determine if the five genetic groups identified in this study exhibit any phenotypic differences that can be attributed to genome variation.

Some of the limitations of RAPD analysis are that the intensities of the bands vary with the quality and quantity of the target DNA, making it difficult to interpret the profiles. Some weak bands may not be visible despite the fact that the target sequence is present in the sample. The profiles can also be influenced by primer specificity, and/or the use of different thermal cyclers, which may result in a lack of reproducibility. Although the limitations of RAPD analysis are numerous, they do not surpass the usefulness of the technique in differentiating species and strains of a range of parasite (Gasser, 2006). Because no previous sequence information is required in the design of arbitrary primers, analysis of RAPDs can be applied on the DNA of organisms whose genomes have not been sequenced. This tool can also be used in genetic and evolutionary studies, and to find new genetic markers (Tibayrenc *et al.,* 1993).

5.2 Conclusion and Recommendations

The tourism sector in South Africa is growing at an escalating rate, accompanied by an increase in game parks that are home to a variety of wild animals. The revenue generated from the tourism sector may be good, but that does not overshadow the negative impact that the game parks can have on neighbouring communities. For example, the Great Limpopo Transfrontier Park, which is a collaborative project between South Africa, Zambia, Zimbabwe and Malawi, allows for unrestricted movement of wild life. The fear of neighbouring livestock keepers is that many diseases that were previously confined to some countries will be spread to other localities. This makes information on epidemiology of diseases such as trypanosomosis essential for the management and control of the disease and its vectors. If control measures are not put in place, the resource poor farmers who reside nearby will continue to suffer while the vectors and reservoirs of various parasites are kept protected.

The following conclusions can be drawn from this study:

- At least two species of trypanosomes are present in KZN, *T. vivax* and *T. congolense*. The two species can occur as mixed infections in the hosts.
- *T. congolense* is the predominant species of trypanosome in KZN.
- There are at least two genotypic groups of *T. congolense* in KZN, the Savannah- and the Kilifi-type. The former is predominant.
- Savannah-type *T. congolense* in KZN are heterogeneous, and there are at least five different genetic groups in the province.

The current situation of *nagana* in South Africa can lead to an outbreak if control measures are not initiated. This study will alert the policy makers of the country of the situation and to take measures that can assist the farmers in maintaining disease free livestock.

Some of the initiatives that are being put in place to assess the problem are the monthly monitoring of sentinel herds as well as the cattle in poor body condition belonging to communal farmers.

There is currently no control policy for tsetse and trypanosomosis in KZN. A long term solution is therefore desirable which should involve the eradication of tsetse flies in the infested areas. The method of diagnosis need to also be improved, one of these is the coupling of more robust DNA extraction methods, with real-time PCR targeting conserved

regions (e.g. the 18S or ITS regions) of DNA that differentiate the different species of trypanosomes.

Given these observations and the limited scope of the current study, a more comprehensive investigation should be undertaken using a larger, widespread (in terms of location) and cloned trypanosome populations. This could rule out any bias that come up as a result of small sample size, confined location and mixed populations.

6. APPENDICES AND REFERENCES

6.1 Appendix 1

6.1.1 Solutions

1M NaCl

NaCl of concentration 1M was prepared by weighing 120g of NaCl in a final volume of 500ml of distilled H_2O.

0.5M EDTA pH 8.0

A stock solution of 0.5M EDTA was prepared by dissolving 93.05g of EDTA in 400ml of ddH_2O. The pH of the solution was adjusted with 5M NaOH to pH 8.0, and the volume was adjusted to 500ml.

5M NaOH

NaOH of concentration 5M was prepared by dissolving 20g of NaOH in a final volume of 100ml of ddH_2O.

0.5M Tris

Tris buffer was prepared by dissolving 30.35g of Tris in 400ml of ddH_2O. The pH was adjusted to 7.4 with 6M HCl, and the volume was adjusted to 500ml.

10% SDS

A 10% SDS solution was made by dissolving 10g of SDS in 100ml of dH_2O.

20% Glycerol

A working stock of 20% glycerol was prepared by diluting 20ml of glycerol in 80ml of dH_2O.

6.1.2 Buffers

NET

To prepare 500ml of NET buffer; 75ml of 1M NaCl, 50ml of 0.5M EDTA and 10ml of 0.5mM Tris were diluted in 365ml of dH_2O.

PBS

To prepare 1L of Phosphate buffered saline, the following salts were dissolved to a final volume of 1L of dH_2O: 13.48g of Na_2HPO_4, 0.78g of $NaH_2PO_4.2H_2O$ and 4.35g of NaCl.

10x TAE

The 10x stock solution of TAE buffer was prepared by weighing and dissolving 48.4g of Tris-base in 750ml of ddH_2O. To this 11.42ml of glacial acetic acid and 20ml of 0.5M EDTA was added and the solution was adjusted to a final volume of 1L. To prepare a working stock of 1x concentration, 100ml of the 10x buffer was diluted to 1L.

10xTBE

The 10x stock solution of TBE buffer was prepared by weighing and dissolving 108g Tris-Base and 55g Boric acid in 750ml of dH_2O. To this, 40ml of 0.5M EDTA solution was added and the solution was adjusted to 1L. To prepare a working stock of 1x concentration, 100ml of the 10x buffer was diluted to a final volume of 1L.

TE-1

To prepare 1L of TE-1 buffer; 10ml of 1M Tris and 200ml of 0.5M EDTA, pH 8 were diluted in 790ml of dH_2O.

6.1.3 Enzymes

RNAse A of concentration 10mg/ml was prepared by dissolving 5mg of pelleted RNase A (Roche, USA, Madison) in 500µl of nuclease free H_2O (Promega Corporation, USA, Madison). The solution was then boiled at 100ºC for 1minute, after which it was immediately transferred to ice, and then stored at -20ºC until further use.

Proteinase K of concentration 10mg/ml was prepared by dissolving 5mg of pelleted Proteinase K recombinant (Roche, USA, Madison) in 500µl of nuclease free H_2O (Promega, South Africa). The enzyme was used immediately or stored at -20ºC until use

6.2 References

Adams, E.R., Malele, I.I., Msangi, A.R., Gibson, W.C., 2006. Trypanosome identification in wild tsetse population in Tanzania using generic primers to amplify the ribosomal RNA ITS-1 region. *Acta Tropica* 100, 103–109.

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, 1–19.

Bruce, D., 1895. Preliminary report on the tsetse fly disease or *nagana* in Zululand. Ubombo, Zululand, December 1895. Durban: Bennet and David

Burudi, E.M.E., Peregrine, A.S., Majiwa, P.A.O., Mbiuki, S.M., Murphy, N.B., 1994. Response of diminazene-resistant and diminazene-susceptible *Trypanosoma congolense* to treatment with diminazene when occurring as a mixed infection in goats. *Annals of Tropical Medicine and Parasitology* 88, 595–606.

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. Oxford University Press 1, 251–296.

Cox, A., Tilley, A., McOdimba, F., Fyfe, J., Eisler, M., Hide, G., Welburn, S., 2005. A PCR based assay for detection and differentiation of African trypanosome species in blood. *Experimental Parasitology* 111, 24–29.

Desquesnes, M., Mclaughlin, G, Zoungrana, A., Davila A.M.R., 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, 609–613.

Dias Neto, E., Caballero, O.L., Vidigal, T.H., Pena, S.D., Simpson, A.J., 1997. Partially degraded DNA of parasitological interest serves as an adequate template for the production of random amplified polymorphic DNAs (RAPDs). *The Journal of Parasitology* 83, 753– 55.

Du Toit, R., 1954. Trypanosomiasis in Zululand and the control of tsetse flies by chemical means. *Onderstepoort Journal of Veterinary Research* 26, 317–387.

Duvallet, G., De La Rocque, S., Reifenberg, J.M., Solano, P., Lefrancois, T., Michel, J.F., Bengaly, Z., Sidibe, I., Cuisance, D., Cuny, G., 1999. Review on the molecular tools for the understanding of the epidemiology of animal trypanosomosis in West Africa. *Memorias do Instituto Oswaldo Cruz* 94, 245–248.

Eisler, M.C., Lessard, P., Masake, R.A., Moloo, S.K., Peregrine, A.S., 1998. Sensitivity and specificity of antigen-capture ELISAs for diagnosis of *Trypanosoma congolense* and *Trypanosoma vivax* in cattle. *Veterinary Parasitology* 79, 187–201.

Eisler, M., 2003. ICPTV Newsletter on integrated control of pathogenic trypanosomes and their vectors. No. 7.

Esterhuizen, J., Kappmeier-Green, K., Nevill, E.M., Van den Bossche, P., 2006. Selective use of odour-baited, insecticide-treated targets to control tsetse flies *Glossina austeni* and *G. brevipalpis* in South Africa. *Medical and Veterinary Entomology* 20, 464–469.

Evans, G., 1880. Report on 'surra' disease in Dera Ismail Khan district. Punjab Government Military Department No 493, 493–4467.

FAO. 2003. Strategic reviews of traps and targets for tsetse and African trypanosomiosis. P9-12.

Gasser, R.B., 2006. Molecular tools–Advances, opportunities and prospects. *Veterinary Parasitology* 136, 69–85.

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, 171–180.

Gibson, W.C., White, T.C., Laird, P.W., Borst, P., 1987. Stable introduction of exogenous DNA into *Trypanosoma brucei. European Molecular Biology Organization Journal* 6, 2457–2461.

Gibson, W.C., Dukes, P., Gashumba, J.K., 1988. Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. *Parasitology* 97, 63–73.

Gibson, W., Stevens, J., 1999. Genetic exchange in the Trypanosomatidae. *Advances in Parasitology,* 43: 1–46.

Gibson, W., Bailey, M., 2003. The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosome. *Kinetoplastid Biology and Disease* 2, 1–13.

Godfrey, D.G., 1961. Types of *Trypanosoma congolense* II. Differences in the courses of infection. *Annals of Tropical Medicine and Parasitology* 55, 154–166.

Gonzalez, A., Prediger, E., Huecas, M.E., Nogueira, N., Lizardi, P., 1984. Minichromosomal repetitive DNA in *Trypanosoma cruzi*: Its use in a high-sensitivity parasite detection assay. *Proceedings of the National Academy of Sciences* 81, 3356–3360.

Hanotte, O., Bradley, D.G., Ochieng, J.W., Verjee, Y., Hill, E.W., Rege, E.O., 2002. African pastoralism: genetic imprints of origins and migrations. *Science* 296, 336–339.

Hendricks, E., Deursen, F.J., Wilson, J., Sarkar, M., Timms, M., Matthwes, K.R., 2000. Life-cycle differentiation in *Trypanosoma brucei*: molecules and mutants. *Biochemical Society Transactions* 28, 531–536.

Herbert, W.J., Lumsden, W.H.R., 1976. *Trypanosoma brucei.* A rapid matching method for estimating the host's parasitemia. *Experimental Parasitology* 40, 427–431.

Jenni, L., Marti, S., Schweizer, J., Betschart, B., Lepage, R.W.F., Well, J.M., Tait, A., Paindavoine, P., Pays, P., 1986. Hybrid formation between African trypanosomes during cyclical transmission. *Nature* 322, 173–175.

Kappmeier, K., Nevill, E.M., Bagnall, R.J., 1998. Review of tsetse flies and trypanosomosis in South Africa. *Onderstepoort Journal of Veterinary Research* 65, 195–203.

Kappmeier, K., Nevill, E.M., 1999. Evaluation of a proposed odour-baited target to control the tsetse flies *Glossina brevipalpis* and *Glossina austeni* (Diptera: Glossinidae) in South Africa. *Onderstepoort Journal of Veterinary Research* 66, 327–332.

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.

Katakura, K., Lubinga, C., Chitambo, H., 1997. Detection of *Trypanosoma congolense* and T. *brucei* subspecies in cattle in Zambia by polymerase chain reaction from blood collected on a filter paper. *Parasitology Research* 83, 241–245.

Kuzoe, F.A.S., Schofield, C.J., 2004. Strategic review of traps and targets for tsetse and African trypanosomiasis control. World Health Organization on behalf of the Special Programme for Research and Training in Tropical Diseases.

Leak, S.G.A., 1998. Tsetse biology and ecology: Their role in the epidemiology and control of trypanosomosis. CABI Publishing in association with the International Livestock Research Institute, Nairobi, Kenya.

Lanar, D.E., Levy, L.S., Manning, J.E., 1981. Complexity and content of the DNA and RNA in *Trypanosoma cruzi. Molecular and Biochemical Parasitology* 3, 327–341.

Lanham, S.M., Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Experimental Parasitology* 28, 521–534.

Majiwa, P.A.O., Young, J.R., Hamers, R., Matthyssens, G., 1986. Minichromosomal variable surface glycoprotein genes and molecular karyotypes of *Trypanosoma (Nannomonas) congolense. Gene* 41, 183–192.

Majiwa, P.A.O., Otieno, L.H., 1990. Recombinant DNA probes reveal simultaneous infection of tsetse flies with different trypanosome species. *Molecular and Biochemical Parasitology* 40, 245–253.

Majiwa, P.A.O., 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.

Masake, R.A., Nyambati, V.M., Nantulya, V.M., Majiwa, P.A.O., Moloo, S.K., Musoke, A.J., 1988. The chromosomes profiles of *Trypanosoma congolense* isolates from Kilifi, Kenya and their relationship to serodeme identity. *Molecular and Biochemical Parasitology* 30, 105–112.

Masake, R.A., Majiwa, P.A.O., Moloo, S.K., Makau, J.M., Njuguna, J.T., Maina, M., Kabata, J., Ole-Moiyoi, O.K., Nantulya, V.M., 1997. Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. *Experimental Parasitology* 85. 193–205.

Mashishi, M.S.K., 2002. Diseases shared by wildlife and livestock. Report for Department of Agriculture. South Africa.

Masiga, D.K., Smyth, A.J., Hayes, P., Bromidge, T.J., Gibson, W.C., 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* 22, 909–918.

Masiga, D.K., McNamara,J.J., Gibson, W.C., 1996. A repetitive DNA sequence specific for *Trypanosoma (Nannomonas) godfreyi. Veterinary Parasitology* 62, 27–33

McNamara, J.J. Mohammed, G., Gibson, W.C., 1994. *Trypanosoma (Nannomonas) godfreyi* sp. nov. from tsetse flies in the Gambia: biological and biochemical characterization. *Parasitology* 109, 497–509.

Mendelman, L.V., Boosalis, M.S., Petruska, J., Goodman, M.F., 1989. Nearest neighbour influences on DNA polymerase insertion fidelity. *Journal of Biological Chemistry* 264, 14415–14423.

Morlais, I., Grebaut, P., Bodo, J.M., Djoha, S., Cuny, G., Herder,S., 1998. Detection and identification of trypanosomes by polymerase chain reaction in wild tsetse flies in Cameroon. *Acta Tropica* 70, 109–117.

Morrison, L.J., Majiwa, P.A.O., Read, A.F., Barry J.D., 2005. Probabilistic order in antigenic variation of *Trypanosoma brucei*. *International Journal for Parasitology* 35, 961–972.

Mugittu, K.N., Silayo, R.S., Majiwa, P.A.O., Kimbita, E.K., Mutayoba, B.M., Maselle, R., 2001. Application of PCR and DNA probes in the characterisation of trypanosomes in the blood of cattle in farms in Morogoro, Tanzania. *Veterinary Parasitology* 94, 177–189.

Njiru, Z.K., Makumi, J.N., Okoth, S., Ndungu, J.M., Gibson, W.C., 2004. Identification of trypanosomes in *Glossina pallidipes* and *G. longipennis* in Kenya. *Infection, Genetics and Evolution* 4, 29–35.

Nyeko, J.H.P., Ole-Moiyoi, O.K., Majiwa, P.A.O., Otieno, L.H., Ociba, P.M., 1990. Characterization of trypanosome isolates from Uganda using species-specific DNA probes reveals predominance of mixed infections. *Insect Science and its Application* 11, 271–280.

OIE, Institute for International Cooperation in Animal Biologics, 2003. Trypanosomiosis. www.who.int/tdr/publications/ publications/pdf/tsetse_traps.pdf.

Okoth, J.O., 1999. Tsetse and trypanosomiasis control problems in South-East Uganda: past, present and alternative strategies. *Schweiz Med Wochenschr* 129, 1091–1098.

Ordner, G., Colardelle, C., D'ieteren, C.D.M., Dumont, P., Itty, P., Jeannin, P., Leak, S.G.A., Maehl, J.H.H., Nagda, S.M., Paling, R.W., Rarieya, J.M., Thorpe, W., Trail , J.C.M., Yangari, G., 1990. Health and productivity of trypanotolerant and susceptible cattle exposed to

trypanosomiasis in Gabon and the impact of strategic chemoprophylaxis. www.fao.org/Wairdocs/ILRI/x5443e0.htm.

Peregrine, A.S., 1994. Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology* 54, 223–248.

Phelps, R.J., Lovemore, D.F., 1994. Vectors: tsetse flies. In: Coetzer, J.A.W., Tustin, R.C. (Eds). Infectious diseases of livestock 2nd edition. Oxford University Press

Rebeski, D.E., Winger, E.M., Okoro, H., Kowalik, S., Burger, H.J., Walter, D.E., Robinson, M.M., Dwinger, R.H., Crowther, J.R. 2000. detection of *Trypanosoma congolense* antibodies with indirect ELISAs using antigen-precoated micotitre plates. *Veterinary Parasitology* 89, 187–198.

Sekoni, V.O., Kumi-Diaka, J., Saror, D., Njoku, C., 1988. The effects of *Trypanosoma vivax* and *Trypanosoma congolense* infections on the reaction time and semen characteristics in the Zebu bull. *British Veterinary Journal* 144, 388–395.

Sekoni, V.O., 1994. Reproductive disorders caused by animal trypanosomiases [sic]: a review. *Theriogenology* 42, 557–570.

Slippers, S.C., 2001. A systems approach to evaluation of feeds and feeding of poultry and small ruminants on small-scale farms in KwaZulu-Natal. Proceedings of Animal Production Research in KwaZulu-Natal, KwaZulu-Natal Branch Symposium, 24 July 2001.

Sloof, P., Bos, J.L., Konings, A.F., Menke, H.H., Borst, P., Gutteridge, W.E., Leon, W., 1983a. Characterization of satellite DNA in *Trypanosoma brucei* and *Trypanosoma cruzi*. *Journal of Molecular Biology* 167, 1–21.

Sloof, P., Menke, H.H., Caspers, M.P., Borst, P., 1983b. Size fractionation of *Trypanosoma brucei* DNA: localization of the 177bp repeat satellite DNA and a variant surface glycoprotein gene in a mini-chromosomal DNA fraction. *Nucleic Acids Research* 11, 3889–3901.

Stevens, J.R., Brisse, S., 2004. Systematics of typanosomes of medical and veterinary importance. In Mauldlin, I., Holmes, P.H., Miles., M.A. (eds) The Trypanosomiases. CAB International p1–23.

Tait, A., MacLeod, A., Tweedie, A., Masiga, D., Turner, C.M.R., 2007. Genetic exchange in *Trypanosoma brucei* : Evidence for mating prior to metacyclic stage development. *Molecular and Biochemical Parasitology* 151, 131–136.

Tibayrenc, M., Neubauer, K., Barnabe, C., Guerrini, F., 1993. Genetic characterization of six parasitic protozoa: Parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proceedings of the National Academy of Sciences of the United States of America* 90, 1335–1339.

Uilenberg, G. (Ed.), 1998. A field guide for the diagnosis, treatment and prevention of African animal trypanosomiasis. Food and Agriculture Organization of the United Nations

Van den Bossche, P., Akoda, K., Kubi, C., Marcotty, T., 2006a. The transmissibility of *Trypanosoma congolense* seems to be associated with its level of resistance to isomentamidium chloride. *Veterinary Parasitology* 135, 365–367.

Van den Bossche, P., Esterhuizen, J., Nkuna, R., Matjila, T., Penzhorn, B., Geerts, S., Marcotty, T., 2006b. An update of the bovine trypanosomosis situation at the edge of Hluhluwe-Umfolozi Park, KwaZulu-Natal, South Africa. *Onderstepoort Journal of Veterinary Research* 73, 77–79.

Vanhamme, L., Lecordier, L., Pays, E., 2001. Control and function of the bloodstream variant surface glycoprotein expression sites in *Trypanosoma brucei. International Journal for Parasitology* 31, 523–531.

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., 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 sterile insect technique. *Journal of Economic Entomology* 93, 123–35.

Welsh, J., McClelland, M., 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18, 7213–7218.

www.kzntopbusiness.co.za

www.sanbi.org/landdeg/grafics/kwazulunatafs.pdf

Young, C.J., Godfrey, D.G., 1983. Enzyme polymorphism and the distribution of *Trypanosoma congolense* isolates. *Annals of Tropical Medicine and Parasitology* 77, 467–481.