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Diagnosis and mapping of diminazene aceturate resistance in *Trypanosoma congolense*, Broden 1904, strains circulating in cattle in Matutuíne district, Mozambique

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I declare that this dissertation hereby submitted to University of Pretoria for the degree of MSc (Veterinary Science) has not been submitted by me or anyone else for the degree at this or any other University, that is the result of my own work in design and execution and all help and advice, other than that received from tutors, has been acknowledged and primary and secondary sources of information have been properly attributed. Should this statement prove to be untrue, I recognise the right and duty of the Board of Examiners to recommend what action should be taken in line with the University's regulations.

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(Paula Macucule)

Pretoria, 30th March 2014

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ABBREVIATIONS

AAT	African Animal Trypanosomosis
Bp	Base pair
Bw	Body weight
°C	Celsius degrees
DA	Diminazene aceturate
DIGIT	Drug incubation *Glossina* infectivity test
DIIT	Drug incubation infectivity test
DMSO	Dimethylsulfoxide
DNA	Desoxiribonucleic acid
dNTP	Deoxyribonucleotide
EDTA	EthylenediamineTetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram
GIS	Geographical Information System
HAT	Human African Trypanosomosis
HAPT1	High-affinity pentamidine transporter
im	Intramuscular

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ISM	Isometamidium Chloride
kDNA	Kinetoplast desoxiribonucleic acid
kg	Kilogram
km^2	Square kilometer
mg	Milligram
$MgCl_2$	Magnesium Chloride
mM	Millimolar
MT	Mouse test
NaCl	Natrium Chloride
NTD	Neglected Tropical Disease
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction - Restriction Fragment Length Polymorphism
PCV	Packed Cell Volume
PSG	Phosphate Saline Glucose
rDNA	Ribosomal desoxiribonucleic acid
RFLP	Restriction Fragment Length Polymorphism

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RNA	Ribonucleic acid
SNP	Single Nucleotide Polymorphism
SSCP	Single-strand conformation polymorphism
Ssu	Small sub-unit
TBE	Tris Borate EDTA
TDR	Trypanocidal Drug Resistance
Tris-HCl	Tris- Hydrochlorate
U	Enzyme unit
USD	United States Dollars
UV	Ultra violet
v:v	volume:volume
WHO	World Health Organization
%	Percentage
Ml	Microliter
µM	Micromolar

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SUMMARY

Diagnosis and mapping of diminazene aceturate resistance in *Trypanosoma congolense*, Broden 1904, strains circulating in cattle in Matutuíne district, Mozambique

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Trypanocidal Drug Resistance is presently recognized as an important constraint for trypanosomosis control efficiency in the endemic areas. No recent and up to date report is available for the Matutuíne District, which is preventing the establishment of an appropriate control strategy in this endemic area. The present study was carried out in order to assess and map the diminazene resistance in *T. congolense* populations circulating in bovines in the Matutuíne District. Between 2009 and 2011, a longitudinal survey was performed, allowing an updated vision of the disease prevalence and of the drug resistance situation.

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A total of 2427 bovine blood samples were collected on filter paper in all five administrative areas in Matutuine (Belavista, Zitundo, Catuane, Katembe and Machangulo). The parasitological (buffy coat) prevalence was 17.3% while using the molecular test 18S PCR, a prevalence of 46% was recorded. Four trypanosome species were detected: the pathogenic *T. congolense* (savannah and Kilifi type), *T. vivax*, *T. b. brucei* and the non-pathogenic *T. theileri*. The *T. congolense* savannah type was the predominant species with a parasitological prevalence of 12.7% (309) (although including the Kilifi type which was not possible to distinguish by buffy coat) and using the molecular test was 22.3% (540) as single infection; 1.6% (149) as mixed infection with *T. theileri*. These results confirm the Matutuíne District as a trypanosomosis-endemic area and even indicate stable disease status, since the prevalence is almost identical to that reported 13 years before. Additionally, it indicates that the *T. congolense* savannah type remains the main etiologic agent of bovine trypanosomosis in the region.
Concerning the diminazene resistance in Matutuíne District, the 689 *T. congolense* savannah 18S PCR positive samples were submitted to the Ade2 PCR followed by the DpnII-PCR-RFLP. Only 21% (143) of the samples were amplified on the Ade2 PCR, confirming the low sensitivity of this PCR tool compared to the 18S PCR.. Ade2 positive PCR's were from four administrative areas in Matutuíne's i.e. Belavista (55.9%), Zitundo (25.2%), Catuane (15.4%) and Katembe (2.1%). Machangulo was not evaluated.

The DpnII-PCR-RFLP revealed the occurrence of all three possible profiles of the TcoAT1/TcoNT10 gene: sensitive homozygous (17.5%), sensitive heterozygous or mixed (44.8%), and resistant (37.8%). Such TcoAT1/TcoNT10 gene allele distributions have been reported in areas without or with moderate trypanocidal drug usage. Therefore we can assume that this is the status of the Matutuíne District and also that, diminazene resistance is not a major problem in the region, allowing the use of the sanative pair strategy for trypanosomosis control in the area.

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For the evaluation of the correlation between the molecular DpnII-PCR-RFLP and the *in vivo* standardized mouse test for diminazene resistance diagnosis, 24 *T. congolense* savannah type isolates were evaluated by both tests. All of them (24/24) were sensitive to the drug at the dose of 10 mg/kg and 20 mg/kg bw in the in vivo drug sensitivity mouse test with the relapses being checked only by parasitological methods. The molecular test revealed 5/24 homozygous sensitive, 8/24 heterozygous sensitive and 11/24 homozygous resistant isolates.. Based on these data, the K statistic revealed a slight agreement between both tests (K= 0.15), supporting the idea of possibly misleading information in the drug sensitivity mouse test when the relapses are detected by parasitological methods. The molecular tool for detection of resistance to diminazene should not be used at the individual level but rather considering the distribution of the resistance alleles as indicator of drug use intensity. This correlation should be further explored. However, the result of the mouse test also indicates that diminazene resistance in this region has not yet been established.

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CHAPTER I: LITERATURE REVIEW

1.1 Introduction

Trypanocidal Drug Resistance (TDR) is presently recognized as a major constraint to livestock production in tsetse-infested areas. It has been reported in 21 African countries including Mozambique (Chitanga *et al.* 2011; Delespaux *et al.* 2008a). TDR was experimentally confirmed in the Central region of Mozambique (in the Zambézia Province), showing the existence of *Trypanosoma congolense* resistant to Diminazene aceturate (DA) and Isometamidium chloride (ISM), the two main trypanocidal drugs marketed for AAT chemotherapy (Jamal *et al.* 2005).

In Southern Mozambique, the Matutuíne District was recognized as a confined trypanosomosis endemic area more than a century ago, being infested by *Glossina austeni* and *G. brevipalpis* (Dos Santos Dias 1962). Five species of trypanosomes have been reported in Matutuine's livestock: the pathogenic *Trypanosoma congolense*, *T. vivax, T. brucei* and *T. simiae* and the non-pathogenic *T. theileri*. *T. congolense* is described as the most abundant species in cattle, with a seroprevalence of about 75.5%, in the last transversal survey using an ELISA test (Sigauque *et al.* 2000).

The livestock in Matutuíne District have been exposed to trypanocidal drugs for more than 50 years; particularly to DA, which is the most frequently used drug for the treatment of trypanosomosis as well as for babesiosis. All these facts brought up the necessity to conduct a large-scale study looking at the present TDR status in Matutuíne District. This will generate useful information on the development and implementation of sustainable trypanosomosis control strategies for the region.

In Africa, large-scale TDR surveys are scanty. This can be explained by difficulties in the diagnosis, since the existing conventional techniques are expensive, laborious and time-consuming. The recently developed molecular assay for the diagnosis of diminazene resistance in *Trypanosoma congolense* (Delespaux *et al.*

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2008a) is considered as a promising tool with potential to overcome the conventional tests' constraints. This molecular test was developed on the basis of a Single Nucleotide Polymorphism (SNP) in the incorrectly called *TcoAT1* gene, considered at that time as an orthologue of the*TbAT1* gene of *T. brucei brucei*, that encodes for the P2 type purine transporter responsible for the translocation of DA molecules in the trypanosome (Delespaux *et al.* 2006). More recent studies on the *TcoAT1* gene demonstrated that the gene is actually an orthologue of the *TbNT10* gene of *T. b. brucei* and encodes for a P1 type purine transporter (Munday *et al.* 2013). Therefore, *TcoAT1* gene is not involved in the DA transport in *T. congolense*. However, considering the good statistical correlation between the presence of the mutation and a resistant phenotype when using the standardized test in mice as standard in the previous studies (Delespaux *et al.* 2008b; Delespaux *et al.* 2006), a genetic linkage to a resistance determinant cannot be excluded (Munday *et al.* 2013).

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1.2 Trypanosomosis

Trypanosomosis is a disease complex that affects most vertebrates including humans. There are two types of trypanosomosis in Africa, classified according to their hosts: Human African Trypanosomiasis (HAT) or sleeping sickness that affects humans and African Animal Trypanosomosis (AAT) or Nagana that affects domestic vertebrates (Uilenberg 1998). In Africa, trypanosomosis is endemic in the sub-Saharan region between parallels 10 North and 26 South covering an area of approximately 10 million km^2 that correspond to a total of 37 countries (Fig. 1) (Maudlin *et al.* 2004). In Mozambique, only approximately one third of the territory is considered free of the disease (Fig 2).

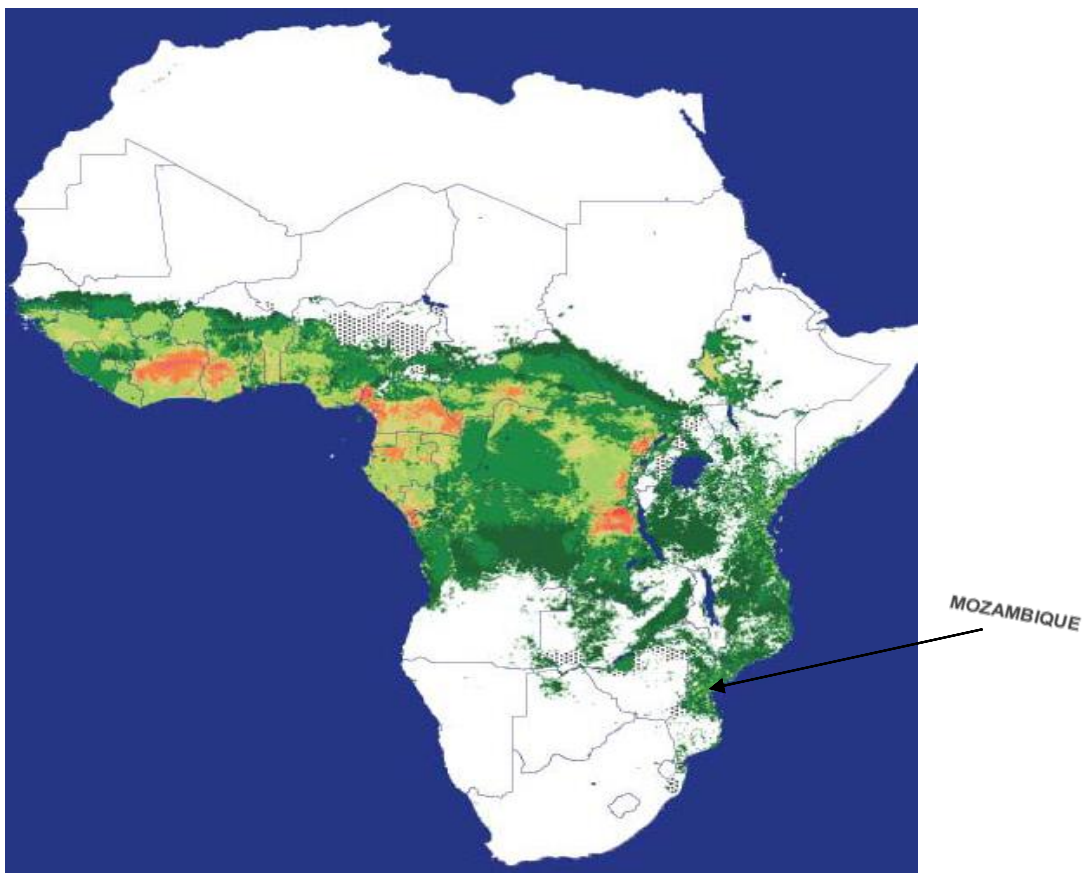

Figure 1: Tsetse fly infested area in Africa. The distribution of trypanosomosis is closely associated with that of its *Glossina* spp. vectors. Mozambique is the southern-most part of that infested area.

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In Mozambique, it appears that tsetse fly and trypanosomosis distribution has a great influence on cattle distribution. The major trypanosomosis-endemic area covers almost all the Central and North regions of the country (Fig. 2). In the south the presently recognized endemic area is confined to the Matutuíne District in Maputo Province (Sigauque *et al.* 2000). In this last region the domestic and wild animals are permanently exposed to *G. brevipalpis* and *G. austeni* challenge.

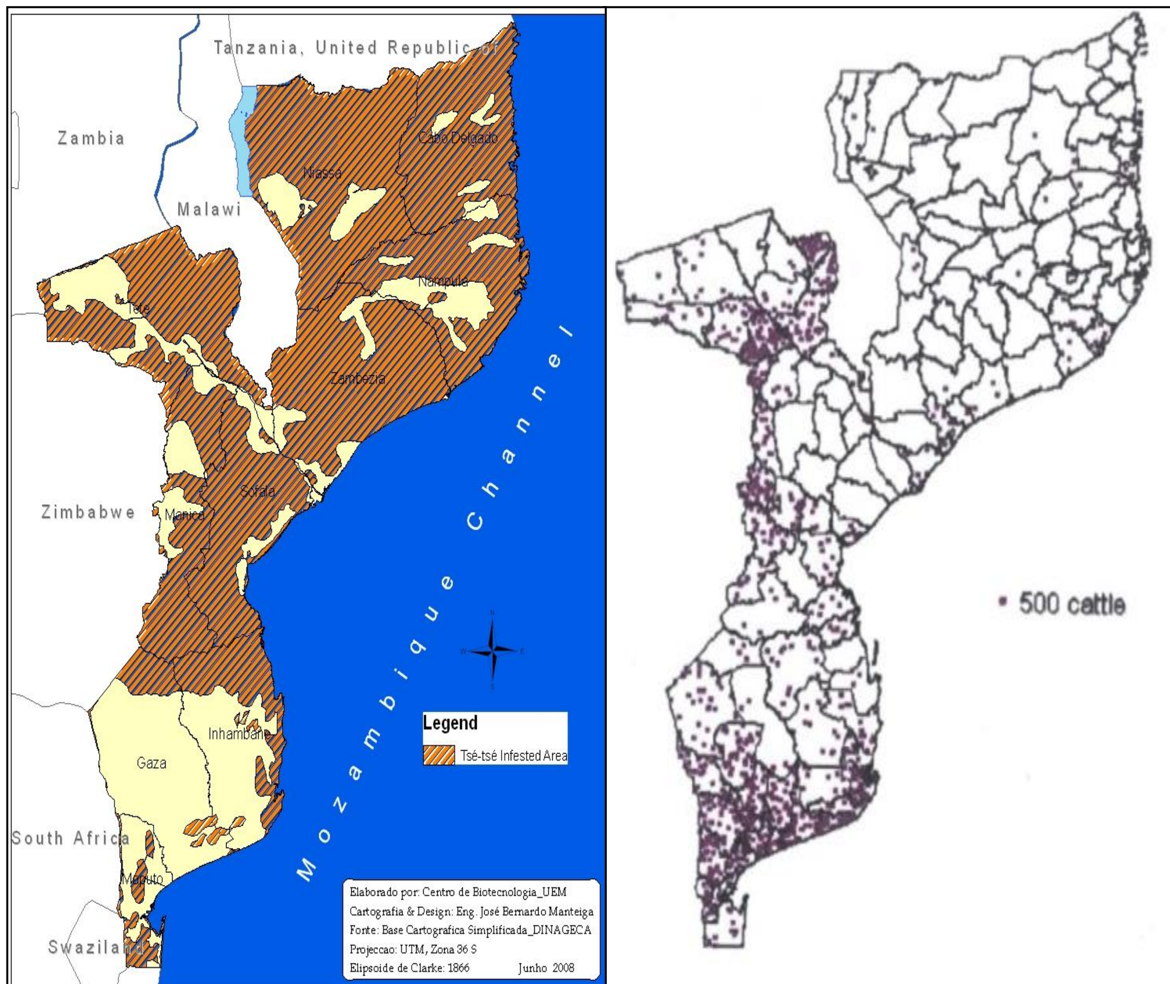

Figure 2: Tsetse fly and cattle distribution in Mozambique. It appear that tsetse fly distribution has a great influence on cattle distribution in all Mozambican territory, since the higher densities of cattle are seen in regions where there are no tsetse flies.

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1.2.1 Trypanosome biology and systematics

Trypanosomes are the causative agents of trypanosomosis. Their body is unicellular and end-pointed (Fig. 3), characterized by the presence of a distinctive DNA mass at the cell's posterior extremity, the kinetoplast DNA (kDNA), which leads to their taxonomic classification into the Order Kinetoplastida (Uilenberg 1998, Maudlin *et al.* 2004). The kDNA encodes small guide RNAs that control the editing specificity as well as ribosomal RNAs and a few mitochondrial proteins similar in structure and genetic function to the mitochondrial DNA of other eukaryotes (Shapiro and Englund 1995).

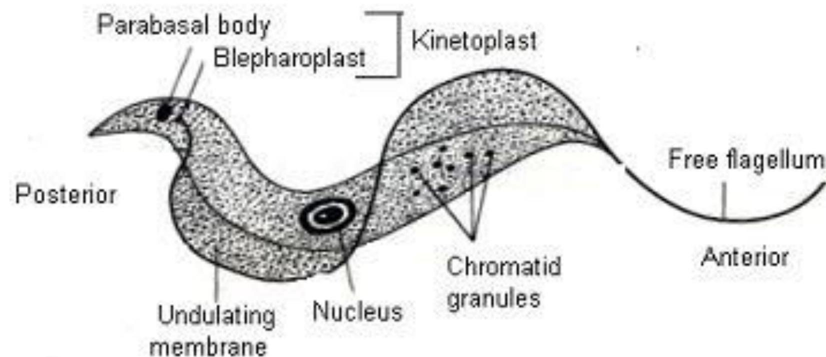

Figure 3: General characteristics of the Trypanosoma spp. cell.

The trypanosomes infecting mammals can only survive in blood, body fluids and tissues of animal hosts. They also have to complete their life cycle inside an arthropod vector (Fig. 4) (Uilenberg 1998, Maudlin *et al.* 2004). For the African trypanosomes, the main vectors are the *Glossina* spp. (commonly called tsetse fly), which are actually the only cyclic vectors. The family Tabanidae and the genus *Stomoxys* (Uilenberg 1998) are described as mechanical vectors (Uilenberg 1998).

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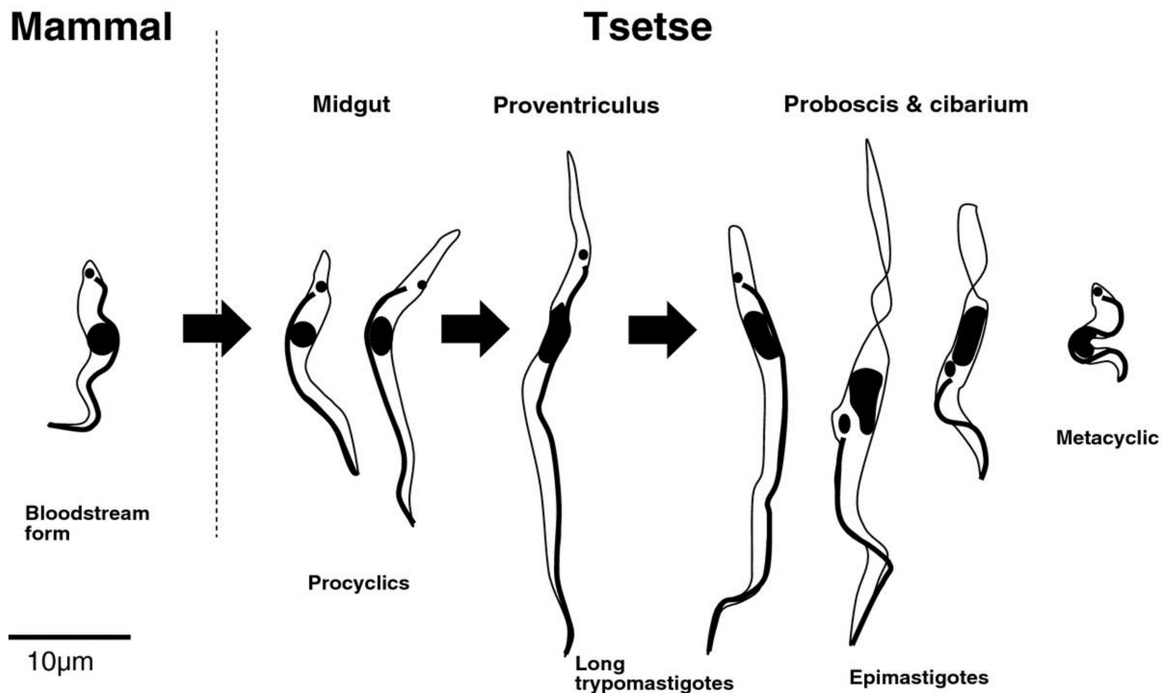

Figure 4: *Trypanosoma congolense* life cycle. "Representative life cycle stages are shown in their respective locations in the mammalian or tsetse hosts". (Peacock *et al.* 2012)

Normally, wild vertebrates are trypanosome reservoirs, as they can live with the parasite for a long time without any clinical disease symptoms (Uilenberg 1998). Conversely, in domestic animals the infection usually results in morbidity and mortality when not treated.

Prior to the advent of molecular tools for trypanosome identification, their taxonomy was based on characteristics related to morphology, kinetics and clinical observations, such as, host range, disease pathology and also on geographical range (Uilenberg 1998). Presently, , the trypanosomes are grouped in the Order Kinetoplastida, Family Trypanosomatidae, Genus *Trypanosoma* (Maudlin *et al.* 2004; Uilenberg, 1998).

The mammalian trypanosomes are divided into Salivaria and Stercoraria sections, according to the mean of transmission by the vector. In the Stercoraria section,

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trypanosomes characteristically develop the infective stage in the vector hindgut and transmission occurs through contamination of the wound by the faeces of the vector. Conversely, the Salivaria species show a transmission by the inoculation by the mouth pieces of the fly and are distributed into three biological groups or subgenera: the subgenus *Trypanozoon* Lühe, 1906, *Dutonella* Chalmers, 1918 and *Nannomonas,* Hoare, 1964 (Uilenberg 1998, Maudlin *et al.* 2004).

The *Trypanozoon* group comprises three morphologically identical subspecies: *Trypanosoma brucei brucei*, which primarily infects cattle, goats and sheep, the *T. brucei gambiense* and *T. brucei rhodesiense* which infect humans and occurs in West and Central Africa and in Eastern and Southern Africa, respectively. The two other groups, *Dutonella* and *Nannomonas*, are represented by species of veterinary interest, *T. vivax* and *T. congolense* being the most important species of either group, respectively. The *T. simae* also belongs to the *Nannomonas* group and is the species responsible for the most acute type of AAT, affecting mainly pigs (Murray *et al.* 1977, Uilenberg 1998).

1.2.2 *Trypanosoma congolense*

Trypanosoma congolense Broden, 1904 (Fig 5), as well as other *Nannomonas*, are small, measuring 8 – 24 µm in length. Three types of genetically different *T. congolense* are presently recognized: Savannah, Forest and Kilifi (Uilenberg 1998). These *T. congolense* sub-types can only be distinguished by molecular-based assays.

T. congolense is mainly transmitted by tsetse flies and shows a broad range of hosts, such as, cattle, goats, sheep and even dogs. However it is considered to be mostly related to the cattle infection. Amongst the three types of *T. congolense*, the most pathogenic in cattle is the savannah type (Van den Bossche *et al.* 2011). Actually, *T. congolense* has been reported as the most abundant specie in cattle in

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Matutuíne District (Sigauque *et al.* 2000) and KwaZulu-Natal (Gillingwater *et al.* 2010; Mamabolo *et al.* 2009) the southernost trypanosomosis-endemic regions of Africa.

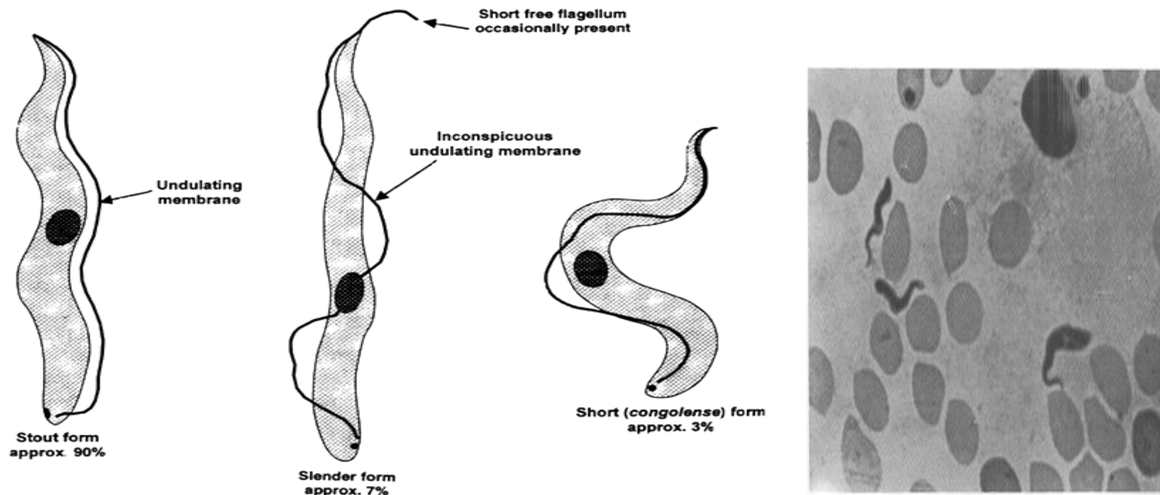

Figure 5: *Trypanosoma congolense* body characteristics. *Trypanosoma congolense* normally lacks a free flagellum and has an inconspicuous undulating membrane. The kinetoplast is marginal and sub-terminally located.

1.2.3 African Animal Trypanosomosis impact

Trypanosomosis is classified by the World Health Organization (WHO), as one of the 11 most important Neglected Tropical Diseases (NTDs). The disease constitutes a serious constraint for the development of many African countries. AAT is the main obstacle for the viability of the animal husbandry systems with direct impact on the human economic and social livelihoods in those countries.

AAT is typically a sub-acute or chronic disease, characterized by anaemia, weight loss, progressive emaciation and enlargement of the peripheral lymph nodes. The disease can lead to death if a proper treatment is not administered. In domestic animals, the disease is strongly associated with morbidity, mortality and reduction in productivity (Murray *et al.* 1977).

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In Africa, losses in meat and milk production as well as, animal traction due to trypanosomosis, are in the order of millions United States Dollars (USD) per year. Additionally, if the indirect loss in agricultural and livestock production is considered, the estimated value of losses will increase to 5 billion USD per year (Anene *et al.* 2001).

1.3 African Animal Trypanosomosis Control

Trypanosomosis control can be done by controlling the vector, controlling the parasite or a combination of both (Delespaux *et al.* 2008a; Geerts & Holmes 1998). In African endemic areas, a considerable part of the economic losses related to the disease are also linked to disease control (Anene *et al.* 2001).

Actually, the vector control by chemical, ecological and biological means or a combination of them might not be fully efficient (Schofield and Maudlin 2001). Many factors contribute to this situation, especially, when this control strategy is applied to large geographic areas, where it has been shown to be logistically complex and economically demanding to deal with (Schofield and Maudlin 2001).

The absence of an efficient immuno-prophylactic alternative, such as a vaccine, renders parasite control by chemoprophylaxis and chemotherapy, the main strategy for trypanosomosis control in Africa (Delespaux *et al.* 2008a; Anene *et al.* 2001; Geerts & Holmes 1998). A wide array of compounds was tested in AAT chemotherapy and chemoprophylaxis. However, there are only a few drugs available today and they have been on the market for more than half a century (Delespaux *et al.* 2008a; Holmes *et al.* 2004; Geerts & Holmes 1998).

In general, ISM is mainly used as a prophylactic drug; Homidium has limited prophylactic properties and is mainly used as a therapeutic drug, while DA is essentially a therapeutic drug (Geerts and Holmes 1998, Holmes *et al.* 2004). In general, millions of trypanocidal doses are administered to livestock every year in

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sub-Saharan Africa. Among these, ISM is the most widely used (40%), followed by Homidium (33%) and DA (26%) (Geerts and Holmes 1998), however, as homidium has been less or even not used because of the mutagenic activity, we can assume that more recent data will show diminazene as the second most used trypanocide.

1.3.1 AAT control in Mozambique

In the past, bush clearing was used for tsetse fly control in Mozambique. However, the practice was rapidly considered ecologically unacceptable because it frequently resulted in game devastation. This first approach for trypanosomosis control in Mozambique was applied more than a hundred years ago.

The first report of chemotherapy applied to trypanosomosis control in Mozambique, dates from 1912 and chemotherapy is, to date, the main trypanosomosis control strategy. The first compound to be tested in trypanosomosis chemotherapy, arsenic acid and it was unsuccessful. In 1923 relative successes with tartar emetic were first reported (Rafael 1959, Silva 1959). Tartar emetic compound was described as an active trypanocide at a dose of 1 to 1.5 mg/kg body weight (bw) administered intravenously, though, only against *T. congolense* and *T. vivax* (Silva 1959). However, a rapid development of resistance to tartar emetic by trypanosomes was observed (Rafael 1959, Silva 1959). After that, a series of new chemical compounds were tested and introduced into the Mozambican market with relative therapeutic success.

Phenantridinium compounds, as Dimidium bromide or Phenantridinium 1553, Ethidium bromide or Homidium, Prothidium or R.D. 2801, were introduced in Mozambique in 1950, 1956 and 1958, respectively (Silva 1959). Diamidine compounds like Berenil (DA or diminazene aceturate) were introduced in Mozambique in 1953 and have since been considered the best chemotherapeutic agents. Antrycide compounds like Methil-sulphate, Prosalt and Prosalt RF (the first

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as a therapeutic and prophylactic drug and the others as prophylactic drugs only), were also used, but they all induced a cross-resistance with Phenantrydinium 1553 (Rafael 1959).

In the 1960's, ISM and Homidium salts were released and immediately adopted in Mozambique. Since then, DA (Berenil®), ISM (Samorin®, Trypamidium® and Veridium®) and Ethidium (Homidium®) have been used for AAT chemotherapy and chemoprophylaxis. Homidium salts have been less used in Mozambique because of their mutagenic activity. The other two compounds have been in regular usage, for more than half a century, particularly DA.

Since the previous century, cohabitation between *G. austeni*, *G. brevipalpis* and livestock in Matutuíne District has been reported (Dos Santos Dias 1962). The previously indicated prevalence in cattle was about 13.9%, with 75.5% of the positive cases being infected by *T. congolense* (Sigauque *et al.* 2000).

Between 1940s and 1970s large-scale tsetse and trypanosomosis surveys were carried out in Mozambique. Those surveys were integrated in the "National Program to Combat Trypanosomiasis", the biggest trypanosomosis program in Mozambique which generated all the data about the distribution of the tsetse flies and trypanosomosis in Mozambique available at the time. Matutuíne was covered by this program with a notable chemoprophylaxis and chemotherapy component, which led to a substantial boost of diminazene usage, also due to its efficacy against babesiosis. Additionally, after the war (1985 - 1994), Matutuíne was restocked with cattle but unfortunately the drug usage was uncontrolled. Therefore, this long-term and uncontrolled use of diminazene might have contributed to widespread occurence of TDR in Matutuíne District.

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1.3.2 Diminazene aceturate as a trypanocide

Diminazene is an aromatic diamidine obtained from Surfen C. The molecule is marketed as the diaceturate salt consisting of two amidinophenyl moieties linked by a triazene bridge (Fig.6), in combination with antipyrine as stabiliser to overcome its short duration stability (Peregrine and Mamman 1993). The formulation is at present marketed under a wide range of trade names: Azidine®, Berenil®, Ganaseg®, Ganasegur® and Veriben®.

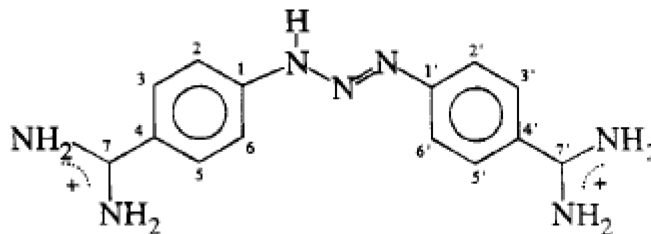

Figure 6: Diminazene aceturate. N-1,3 di-4-amidino-phenyl triazene-diaceturate (source: Peregrine & Mamman, 1993)

Diminazene can provide effective protection to the animal for approximately 2 weeks (Peregrine and Mamman 1993, Holmes *et al.* 2004). This drug has activity against trypanosomes as well as various tick-borne disease parasites including *Babesia* spp. (Holmes *et al.* 2004; Peregrine & Mamman 1993). It's the cheapest and most widely available trypanocidal drug, which makes it frequently the first-line drug for sick cattle in Africa (Peregrine and Mamman 1993, Delespaux and de Koning 2007).

Originally, the treatment of trypanosomosis with diminazene was recommended to be done through intramuscular (im) injection at a dose of 3.5 mg/kg bw to eliminate *T. congolense* and *T. vivax* infections and a dose of 7 mg/kg bw to eliminate *T. brucei* (Peregrine and Mamman 1993). Therefore, because of the rapid clearance of the diminazene molecules (2-7 days), this dosage leads to a very short period of

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drug activity; which can be related with the development of resistance to this trypanocidal drug by the trypanosomes.

1.4 Drug resistance in trypanosomosis

Drug resistance can be defined as a heritable temporary or permanent loss of the initial sensitivity of a microorganism population against an active substance (Schnitzer & Grunberg 1957). In trypanosomosis this phenomenon has been reported since the early stages of chemotherapeutic introduction and lead to the failure of most chemical compounds candidates for chemotherapeutic and/or chemoprophylaxis application. Presently, TDR is reported from 22 of 37 AAT-endemic countries, with the recent addition of Benin, Ghana, Togo and South Africa (Chitanga *et al.* 2011) into the large group of 18 countries where the phenomenon had previously been confirmed (Delespaux *et al.* 2008a). However, it remains unclear whether this growing trend is only because of the emergence of new cases or also due to an apparent new wave of interest in this subject by scientists (Delespaux *et al.* 2008a).

In general, the lack of alternatives to trypanocidal drugs is a major constraint to trypanosomosis control. This leads to an intensive and abusive usage of the three trypanocidal drugs (ISM, DA and Homidium), which is indicated as leading to the rise and widespread occurrence of trypanocide resistance (Geerts and Holmes 1998).

In Mozambique, the occurrence of resistance of *T. congolense* strains to ISM and DA was confirmed in Zambézia Province, using a standard test in mice (Jamal *et al.* 2005).

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1.4.1 *Trypanosoma congolense* diminazene resistance

The mechanism of diminazene resistance in *T. congolense* remains unclear. Conversely, for *T. b. brucei* it is now evident that diminazene molecules are primarily accumulated through a P2 type purine transporter, the *TbAT1* (Matovu *et al.* 2003, Koning *et al.* 2004, Stewart *et al.* 2010). Though this is not the exclusive form of diminazene uptake by this trypanosome species, as the high-affinity pentamidine transporter (HAPT1) way can also be considered (Bridges *et al.* 2007, Teka *et al.* 2011). It is also clear that *TbAT1* transporter is closely related to *T. brucei* diminazene resistance, and that for this trypanosome specie resistance is induced by the loss of *TbAT1* transporter (Bridges *et al.* 2007).

A similar diminazene resistance mechanism was proposed for *T. congolense*. The *T. congolense* putative P2-type purine transporter gene was identified by reciprocal blasting of *TbAT1* gene and being considered an orthologue gene, was named *TcoAT1* (Delespaux *et al.* 2006).

1.4.2 *T. congolense* DA resistance diagnosis

Several tests have been described for the detection of drug resistance in pathogenic trypanosomes. Those which are mostly used are the standardized in vivo tests in mice or livestock, followed by the *in vitro* tests like the drug incubation *Glossina* infectivity test (DIGIT) or drug incubation infectivity test (DIIT) and ELISA.

On the *in vivo* tests, after growth of an isolate in a mouse the trypanosomes are inoculated in experimental animals. Twenty-four hours later for mice or at the first peak of parasitaemia for ruminants, each experimental group except the controls is treated with one or a range of drug doses. Thereafter, the animals should be monitored twice per week for 60 days. The effective dose that gives temporary clearance of the parasites in 50 or 95 percent of the tested animals, (ED50 or ED95 respectively) as well as the curative dose that gives complete cure in 50 or

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95 percent of the animals can be (CD50 or CD95 respectively) is calculated accordingly with the number of relapses (Eisler *et al.* 2001).

In the *in vitro* tests the metacyclic or bloodstream trypanosome forms have to be incubated for 24 h in culture medium in the presence of a range of drug concentrations. Later, these trypanosomes are inoculated in mice for the DIIT test or feed *Glossina* fly in the DIGIT test to assess their infectivity. The mice is then screened for 20-30 days for appearance of parasites (Kaminsky and Brun 1993), while the fly have to be dissected after almost same time (Clausen *et al.* 1999).

All those techniques have high costs as well as being laborious and time-consuming to perform. This restricts their use in epidemiological studies (Delespaux *et al.* 2008a; Anene *et al.* 2001; Geerts & Holmes 1998). Molecular tools give more rapid and convenient results and are considered very useful in large-scale epidemiological studies.

The proposed mechanism of *T. congolense* diminazene resistance was essential to the development of a molecular diagnostic assay. From there, single-strand conformation polymorphism (SSCP) was used to screen sensitive and resistant strains for potential conserved mutation in the resistant phenotype. The target gene was the purine transporter *Tco*AT1/*Tco*NT10.This allowed the development of a rapid diagnostic tool based on PCR-RFLP (Delespaux *et al.* 2008a; Delespaux *et al.* 2006). The technique was used in the recent drug resistance reports (Moti *et al.* 2012; Chitanga *et al.* 2011; Mamoudou *et al.* 2008; Delespaux *et al.* 2008b), and has shown a good correlation with the standardized test in mice.

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1.5 Objectives

The aim of the present research is to assess and map the TDR situation in *T. congolense* isolates circulating in bovines of Matutuíne District, Maputo Province., In all five administrative areas of Matutuíne, the diminazene aceturate sensitivity of the bovine *T.congolense* isolates was surveyed by standardized in vivo mouse test and molecular DpnII-PCR-RFLP. For that, a survey was firstly conducted, looking at the present trypanosomosis status of the district's cattle. The trypanosome species present in bovines was diagnosed by the parasitological test buffy coat and by the 18s-PCR and the MspI-PCR-RFLP

In order to determine the status of the Matutuíne district in terms of TDR, the proportion of the mutated *Tco*AT1/*Tco*NT10 allele was determined by the molecular test DpnII-PCR-RFLP and the district status determined accordingly as well as the distribution mapping.

In order to validate the molecular test, a correlation with the in vivo mouse test was evaluated by testing by both tests 24 isolates of *T. congolense* collected in Matutuíne's bovines. The correlation (Kappa Statistic) between the tests was calculated.

1.6 Potential benefits arising from the research study

This survey about the resistance of *T. congolense* to diminazene may generate crucial information to advise policy makers and the veterinary services in order to develop and implement recommendations on trypanocidal drug usage and rational tsetse and trypanosomosis control in Matutuíne District. Additionally, the study represents the first approach of this nature to be conducted in a trypanosomosis-endemic area in Mozambique and is potentially replicable in other regions of the country.

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The validation of the molecular tool for the diagnosis of DA resistance in *T. congolense*, DpnII-PCR-RFLP, by comparing its results with the standardized test in mice is presently very important, when considering the last scientific indications that the *TcoAT1* transporter is unable to mediate diminazene translocation in *T. congolense* since it is a P1-type purine transporter.

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CHAPTER II: MATERIALS AND METHODS

2.1 Study area

The study was carried out in Matutuíne District, Maputo Province, in Southern Mozambique (Fig. 7). Matutuíne is one of the seven Districts that constitute the Maputo Province. Matutuíne District has been recognized as endemic for trypanosomosis for more than a century (Dos Santos Dias 1962, Sigauque *et al.* 2000). The District is situated in the most south-eastern part of the province extending from 26° 00´ to 26° 50´ S and 32° 00´ to 33° 00´ E. It is limited by the Indian Ocean in the East; the Kingdom of Swaziland, Namaacha District and Boane District in the West; Maputo city in the North and the Republic of South Africa in the South (MINADE 2005).

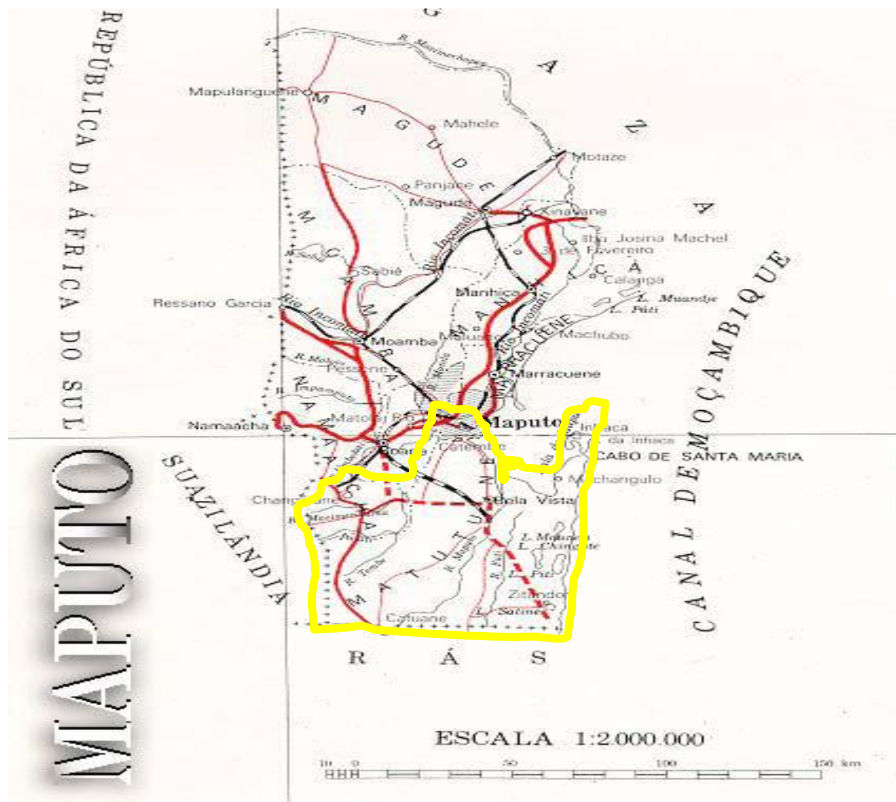

Figure 7: Matutuíne District location on the Maputo Province map (source: MINADE 2005).

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Matutuíne has a total surface of 5 403 km². About 700 km² of this territory belongs to a protected area, the Maputo Game Reserve. So, Matutuíne is an important region in terms of cohabitation of wild and domestic animals. The District is divided into five administrative areas, namely: Catuane, Katembe, Bela-Vista, Zitundo and Machangulo. The total human population recorded is 37 165 inhabitants (MINADE 2005) with about 80% of the active inhabitants directly involved in a low-input subsistence agriculture system, the main economic activity.

The climate and vegetation in Matutuíne are sub-tropical with two main seasons: the hot wet season lasting from November to March with temperatures ranging from 25 °C to 40 °C and the cool dry season lasting from April to October with temperatures ranging from 12 °C to 26 °C. The annual rainfall is about 750 mm. The vegetation is of the savannah type, dominated by *Acacia* spp, *Terminalia sericia*, *Dichrostachys cinerea*, *Euclea natalensis*, *Diospyros mespiliformis* and *Albizia* spp.

Some wild animal species can be found in Maputo Special Reserve: steinbok (*Raphicerus campestris*), common gray duiker (*Sylvicapra grimmia*), reedbuck (*Redunca arundinum*), suni (*Neotragus moschatus*), African elephant (*Loxodonta africana*), hippopotamus (*Hippopotamus amphibius*), impala (*Aepyceros melampus*), bush buck (*Tragelaphus scriptus*), red duiker (*Cephalophus natalensis*), nyala (*Tragelaphus angasi*) and bush pig (*Potamochoerus porcus*) (MINADE 2005).

2.2 Sampling in cattle

In each geo-referenced sampling site, a random sample was taken and the number of samples was calculated to provide (95%) certainty of detecting at least one *T. congolense* positive case at a prevalence of 5% (Cannon and Roe 1982). The

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code, age and sex of each animal sampled were recorded as well as the geographic coordinates of the sampling site.

For each animal, blood was collected from the tail vein into a vacutainer tube with di-sodium salt of ethylene diamine tetra-acetate (EDTA). Packed cell volume (PCV) was recorded and trypanosomes searched for by Buffy Coat method as described by Murray *et al.* (1977). Four supplementary Buffy Coats from capillary tubes without anticoagulant were extruded onto a filter paper (Whatman n° 4, Whatman®). These filter papers were dried, protected from UV light (sunlight) and stored in sealed plastic bags containing silica gel until transfer to the laboratory for further molecular analysis.

From *T. congolense* positive blood samples 0.5 ml of blood was inoculated intraperitoneally into 2 mice (OF1) aged 5-8 weeks, weighing on average 30 g. After three days the parasitaemia was determined daily by the Buffy Coat method. When parasitaemia reached a minimum of 8.1 on the Herbert and Lumsden scale (Herbert and Lumsden 1976) (Annexure A), mice were euthanized and the blood was collected by heart puncture for the preparation of stabilates (Annexure B) using 1:3 (v:v) of a DMSO/PSG 20% solution as cryopreservative for long-term storage in liquid nitrogen or until the *in vivo* Mouse Test was performed.

2.3 Laboratory analysis

2.3.1 The *in vivo* Mouse Test

T. congolense isolates were evaluated for DA resistance using the test in mice in accordance with the protocol described by Eisler *et al.* (2001) (Annexure C). For each trypanosome isolate, 10^5 trypanosomes were intraperitoneally inoculated into three groups of six f 8–10 weeks old mice, weighing 25–30 g. Twenty-four hours after inoculation, two mice groups were treated with (i) 10 mg/kg and (ii) 20 mg/kg body weight (bw) DA (Berenil®, Hoechst AG, Germany 01W005), respectively; the

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third (non treated) was a control group. The mice were monitored twice a week for a period of 60 days for the presence of trypanosomes through the examination of wet smears of tail blood. An isolate was considered resistant when more than one out of the six mice treated with a certain dose became positive within the observation period.

All mice found with high parasitaemia (minimum of 7 on the Herbert and Lumsden scale (Herbert and Lumsden 1976) were removed from the experiment and euthanized. The infected blood was collected by heart puncture. Four spots each containing approximately 60 µl of the parasitaemic blood were placed on Whatman n° 4 filter paper, dried and conserved in silica gel for molecular testing (further described). The extra blood was used for the preparation of stabilates using 1:3 (v:v) of a DMSO/PSG 20% solution as a cryoprotectant (Annex B) for long-term storage in liquid nitrogen.

2.3.2 DNA extraction for molecular tests

Genomic DNA was extracted from the blood dried on the filter paper spots. Each spot was cut in small pieces of 1 mm diameter and placed in a 1.5 µl microcentrifuge tube. Between each sample the puncher was sterilized in a flame. For cell lysis and protein denaturation 200 µl of lysis buffer (50 mM NaCl; 50 mM Tris-HCl pH 7.4; 10 mM EDTA pH 8; 1% Triton X100) and 200 µg/ml Proteinase K were added. The mixture was incubated overnight at 56-60 ºC. Subsequently the samples were washed with an equal volume of Phenol-chloroform (1:1; v: v); Chloroform-Isoamyl Alcohol (24:1); and Chloroform in last. The DNA was then precipitated with 10% of 3M sodium acetate pH 7 and 2 volumes of isopropanol either overnight at -20°C or 30 minutes at -80°C, followed by centrifugation at 12 000 rpm for 30 minutes. The supernatant was discarded and DNA dried at room temperature for further elution in 50 µl TE (10 mM Tris-HCl pH 7.4; 1 mM EDTA pH8) and stored at -20 °C until use.

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2.3.3 DNA amplification for species identification

For species detection and identification, the semi-nested 18S PCR for Ssu-rDNA amplification was performed (Geysen *et al.* 2003). A standard PCR amplification was carried out in 25 µl mix containing 5 µl of DNA sample and 20 µl of PCR mix (1 µl of Yellow Sub®, 1x PCR buffer, 1.65 mM $MgCl_2$, 200 µM of each dNTP, and 10 pmol of each primer and 1U of Taq Polymerase enzyme (Fermentas®). The reaction mixtures was placed on a heating block of a programmable thermocycler with hot start principle where after a denaturation step of 4 min at 94 ºC each of the 40 cycles were done as follows: 30 s at 94 ºC, 45 s at 59 ºC and 60s at 72 ºC. At the end a final elongation step was allowed for 10 min at 72 ºC. For semi-nested runs, 0.5 µl of the first run was added to 24.5 µl of the second run PCR mix containing the same ingredients and concentrations except the primer pair. The amplification program was identical except for 25 cycles.

The first round PCR was done using the forward primer 18STnF2 (CAACGATGACACCCATGAATTGGGGA) and the reverse primer 18STnF3 (TGCGCGACCAATAATTGCAATAC). The second round amplification was done using the forward primer 18STnF2 and the reverse primer 18STnR2 (GTGTCTTGTTCTCACTGACATTGTAGTG). A pair of controls was added, one negative consisting of ultrapure water and one positive consisting of a well characterized positive *T.* congolense DNA.

The semi-nested PCR samples were examined for the presence of DNA amplicons by agarose gel electrophoresis (Annexure D). All positive samples in this step were considered to be *Trypanosoma* positive samples and RFLP was further used for species identification.

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2.3.4 *Msp*I-PCR-RFLP for *Trypanosoma* species identification

The semi-nested PCR products were digested with *Msp*I enzyme in buffer Nº4 following the manufacturer's instructions. In a total volume of 15 µl containing 4 µl amplicon, 1.5 µl 10x buffer N4 and 0.3 µl of 20 U/µl *MspI* enzyme were incubated overnight at 37 ºC. A volume of 4 µl of restricted sample was then analyzed by electrophoresis in a 10% polyacrylamide gel (PAGE) (Annex E) together with a 100 bp DNA ladder. Images were captured under UV illumination. The fragment interpretation and typing was done in accordance with Geysen *et al.* (2003) and Delespaux *et al.* (2003). All *T. congolense* positive samples were further used for DA resistance diagnosis.

2.3.5 DNA amplification for *Trypanosoma congolense* diminazene resistance diagnosis

For diminazene resistance diagnosis a PCR for *TcoAT1*/*TcoNT10* gene amplification was performed according to Delespaux *et al.* (2006). The standard amplification was carried out in a 25 µl volume reaction containing 20 µl of PCR mix (1x PCR buffer, 1.65 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 0.5 U of Taq Polymerase enzyme and 1 µl of Yellow Sub®) and 5 µl of DNA sample (of the *T. congolense* positives on *Msp*I-PCR-RFLP). The reaction mixture was placed on a heating block of a programmable thermocycler with hot start principle where after a denaturation step of 4 min at 94 ºC each of the 40 cycles was done as follows: 30s at 94 ºC, 45s at 59 ºC and 60s at 72 ºC. At the end a final elongation was performed for 10 min at 72 ºC. A pair of controls, one negative consisting of ultrapure water and one positive consisting of a well diminazene resistance status characterized *T. congolense* DNA, were included in all PCR amplifications. The forward primer Ade2F (ATAATCAAAGCTGCCATGGATGAAG) and the reverse primer Ade2R (GTAGACTAACAATATGCGGGCAAAG) were used.

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The samples were then analyzed for the presence of DNA amplicons by agarose gel electrophoresis (Annex D). All *T. congolense* positive samples were further characterized by DpnII-PCR-RFLP for the diagnosis of DA resistance.

2.3.6 DpnII-PCR-RFLP for *Trypanosoma congolense TcoAT1/TcoNT10* allele profile determination

The DpnII-PCR-RFLP was performed as described by Delespaux *et al.* (2006). Ade2 PCR products were digested by DpnII (Fermentas®) enzyme, in a total volume of 15 µl containing 4 µl amplicon, 1.5 µl buffer and 20 U/µl of the enzyme. The reaction was incubated overnight in a water bath at 37 ºC.

A volume of 5 µl restricted sample was then analyzed by electrophoresis in 10% polyacrylamide gel (PAGE) together with a 100 bp DNA ladder and (Annex E). Images were captured under UV illumination. The fragment interpretation and typing were done according to Delespaux *et al.* (2006).

2.4 Ethical statements

Live animals were used for this study. Some were used in laboratory conditions and others in field conditions. Those animals were used without any kind of interference to their normal routine in terms of feeding and water supply.

White mice, with a minimum age of 8 weeks and weighting of 30 g, were obtained from the Biotechnology Centre breeding colony and maintained on an ad libitum supply of a pellet ration and water. The room temperature was kept constant at 25 ºC and good air circulation was maintained.

For all activities with the animals, internationally published or standardized protocols were used in order to promote ethical treatments. After the experiment,

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all live mice were euthanized. All procedures were approved by the Veterinary Faculty ethical committee (Eduardo Mondlane University).

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CHAPTER III: RESULTS

3.1 Sampling

Between March 2009 and December 2012, 75 dip tanks were visited around all five administrative areas of Matutuíne District (Belavista, Zitundo, Catuane, Katembe and Machangulo). From there, 2427 bovine blood samples were collected on filter paper after microscopic analyse by Buffy coat and PCV value records (Fig.8).

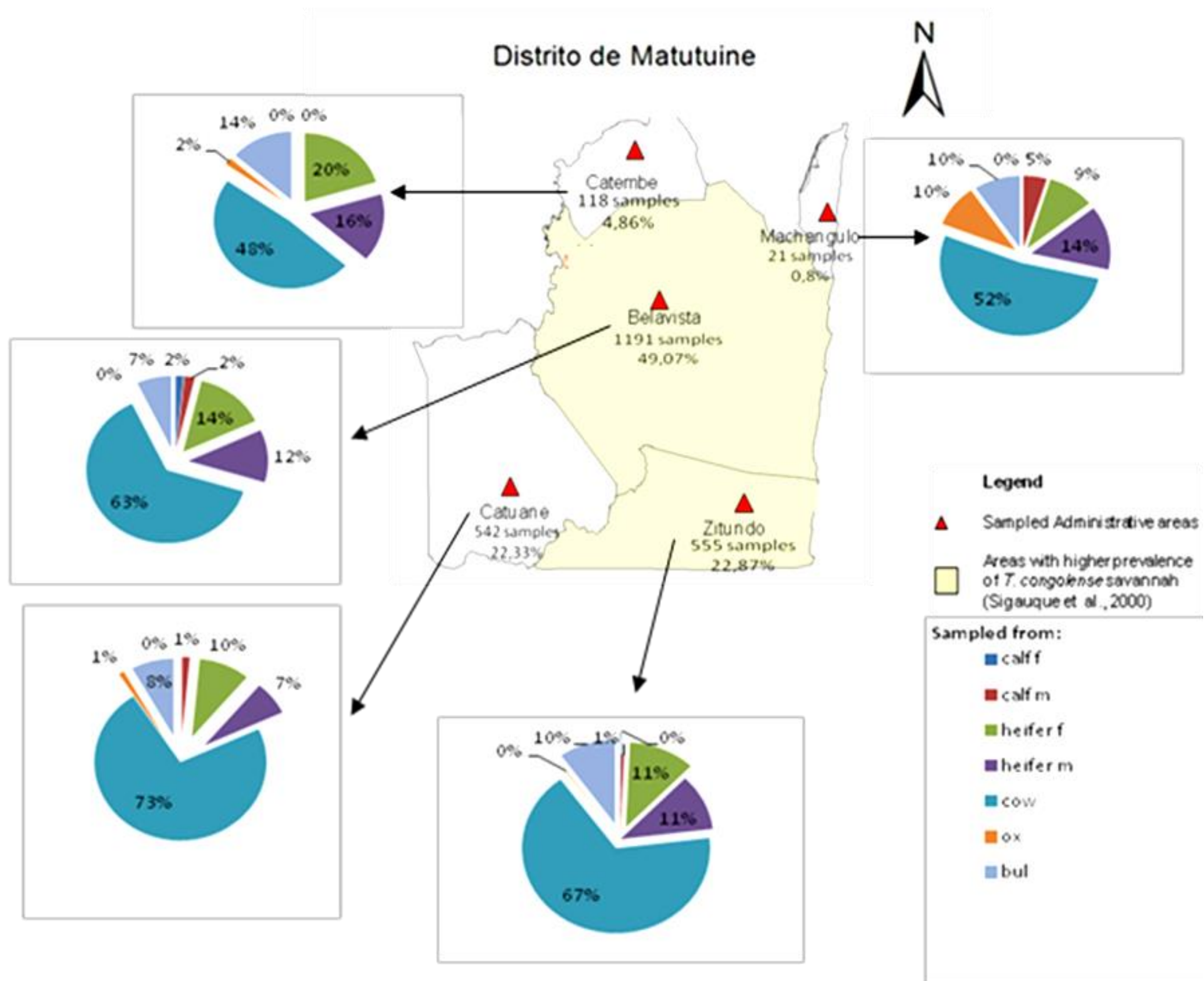

Figure 8: Matutuíne District sampled areas. The most represented areas in the overall sample size (N = 2427) were Belavista (49.07%), Zitundo (22.87%) and Catuane (22.33%). Most of the samples (48–73%) were collected from cows.

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From the 2427 samples, 423 (17.3%) were parasitologically positive for *Trypanosomes*. *T. congolense* occurred as a single infection in 309 (12.7%) samples and also occurred in association with *T. vivax* in 46 samples (1.9%). The others species occurred in lower prevalences: *T. vivax* 30 (1.2%), *T. brucei* 9 (0.4%) and *T. theileri* 29 (1.2%).

From the *T. congolense* single infections, 83 isolates were inoculated in mice from which 25 grew successfully.

3.2 Trypanosome species detection by buffy coat and molecular tests

Table 1: Observed prevalence of trypanosome infections in the five administrative areas of Matutuíne District by parasitological and molecular tests. *The negative variation meaning that the molecular test was less sensitive than the parasitological test

	Observed prevalence of trypanosome infections in bovines (%)					
	Belavista	**Catuane**	**Katembe**	**Zitundo**	**Machangulo**	**Global**
Buffy coat	20.5 (15.2 – 27.1)	5.5 (3.2 – 9.5)	8.8 (2.8 – 15.4)	16.6 (10.1 – 26.0)	0.16	17,35
18S PCR	76.7 (71.3 – 81.4)	8.5 (3.9 – 17.6)	2.5 (0.7 – 9.3)	33.5 (24.1 – 44.4)	0.16	46,0
Variation	**56.2**	**3.0**	**- 6.3***	**16.9**	**0.0**	**28.7**

Trypanosome infections were detected in the five administrative areas of Matutuíne District, either by parasitological test or by molecular testing (18S PCR and *Msp*I–RFLP). The overall prevalence of trypanosome infections was 17.3% by buffy coat and 46.0% by molecular testing. For Belavista, Catuane and Zitundo, molecular testing was more sensitive than the parasitological test in detecting the trypanosome infections (Table 1). In Katembe the molecular test was not able to

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detect 6.3% of the positive parasitological test. Those were *T. vivax* and *T. brucei* infections.

The 18S PCR (Fig. 9) allowed the detection of *T. congolense* savannah type, *T. congolense* kilifi type, *T. vivax, T. brucei brucei* and *T. theileri*.

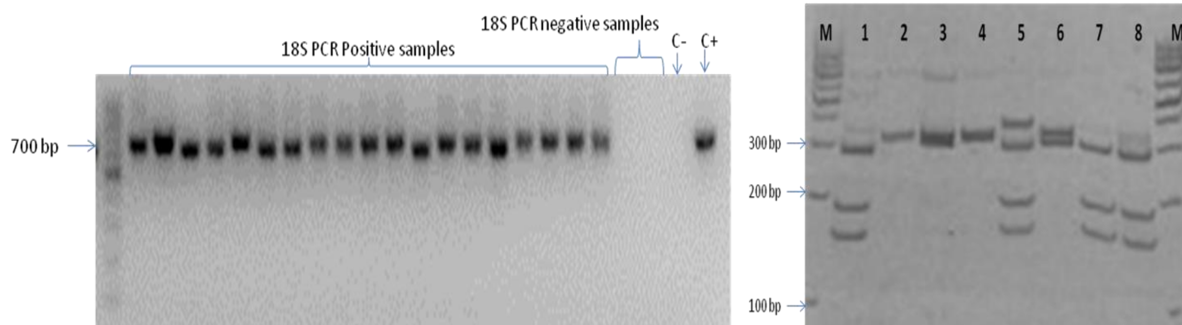

Figure 9: Gel electrophoresis of 18S PCR and MspI – RFLP products. A: Agarose gel electrophoresis for 18S PCR amplicon.B: Polyacrylamide gel electrophoresis for the MspI – RFLP products: *T. congolense* savannah type (line 1 and 7), *T.congolense* kilifi type (line 5), *T. theileri* (line 2; 3; 4 and 6) and positive control IL3000 (line 8)

Notwithstanding the good overall performance of the18S PCR, most of the *T. vivax* infections were not detected either when mixed with *T. congolense* or in single infections (Table 2). In general, this molecular test was four times less sensitive to detect the single *T. vivax* infections (0.3% against 1.2% by the parasitological test). In contrast, the same tool was almost 20 times more sensitive to detect *T. theileri* (16.2% against 1.2%).

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Table 2: The trypanosome species detected by both tests (N=2427).

	Buffy coat		18S PCR	
Detected species	**total**	**%**	**total**	**%**
T. congolense savannah	309	12,7%	540	22,3%
T. congolense kilifi			6	0,2%
T. congolense savannah+ *T. theileri*	0	0,0%	149	6,1%
T. congolense + *T. vivax*	46	1,9%	0	0,0%
T. vivax	30	1,2%	8	0,3%
T. brucei	9	0,4%	16	0,7%
T. brucei + *T. theileri*	0	0,0%	4	0,2%
T. theileri	29	1,2%	394	16,2%
Total positives	**423**	**17,3%**	**1117**	**46,0%**

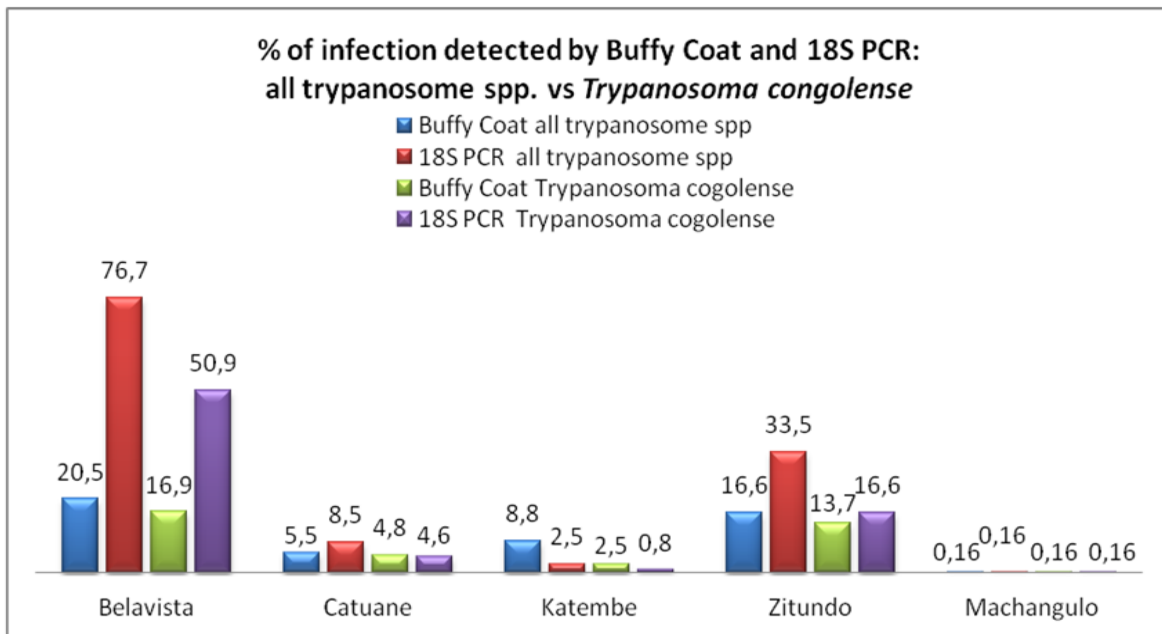

Figure 10: Prevalence of *T.congolense* infections in the five administrative areas in Matutuíne. With the exception of Katembe it was the most prevalent species. At the Katembe, the PCR was less sensitive the buffy coat.

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Most of the infections were by *T. congolense*, which was detected in 83.9% and 62.2% of all positive cases by Buffy coat and 18S PCR, respectively. However, 1.9% of the Buffy Coat positive was in association with *T. vivax*, although these cases were not confirmed by PCR. Actually, *T. congolense* was the most prevalent specie in four administrative areas of Matutuíne (Belavista, Zitundo, Catuane and Machangulo) (Fig. 10). In Katembe, the 18S PCR did not detect some of the cases detected by the parasitological test, i.e 2.5% against 8.8% for all trypanosomes and 0.8% against 2.5% for *T. congolense* infections.

In general, the DNA extracted from the filter paper was low in concentration but of good quality. However, in 5.8 % of the *T. congolense* positive samples by parasitological test, the 18S PCR did not detect this trypanosome species (Table 3), even after DNA re-extraction or amplification. Most of those samples were from Katembe.

Table 3: The *T. congolense* proportions detected by parasitological and molecular testing.

	TC BC -	**TC BC +**	**Total**
TC PCR -	65.7	5.8	**71.5**
TC PCR +	21.5	7.0	**28.5**
Total	**87.1**	**12.9**	**100.00 %**

3.3 PCR Ade2 and DpnII-PCR-RFLP for bovine blood samples

From the 689 *T. congolense* savannah type PCR positive samples, 143 (21%) only were amplified by Ade2 PCR. The amplified samples were from four of the five Matutuíne administrative areas (Belavista, Zitundo Catuane and Katembe) (Fig. 11). Machangulo was not represented in this evaluation.

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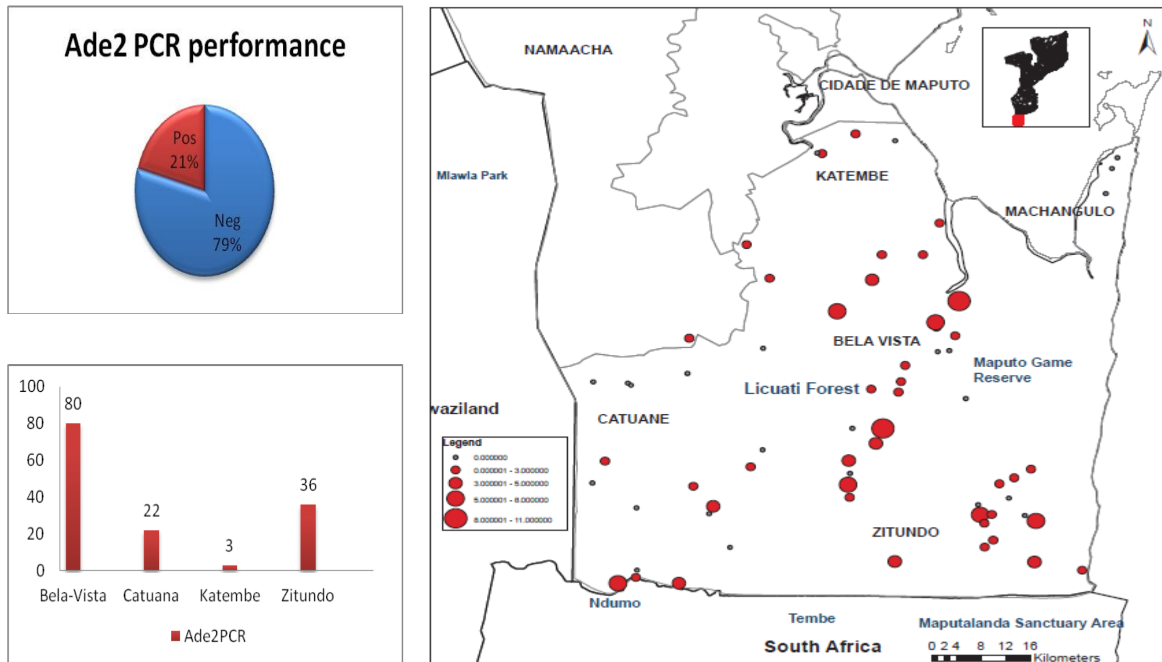

Figure 11: Ade2 PCR performance. For the *T. congolense* savannah positive samples, the Ade2 PCR was able to amplify only 21% samples (top left up). Belavista was the most represented with 55.9% of the samples followed by Zitundo (25.2%), Catuane (15.4%) and Katembe (2.1%) (left down). Machangulo was not represented (right).

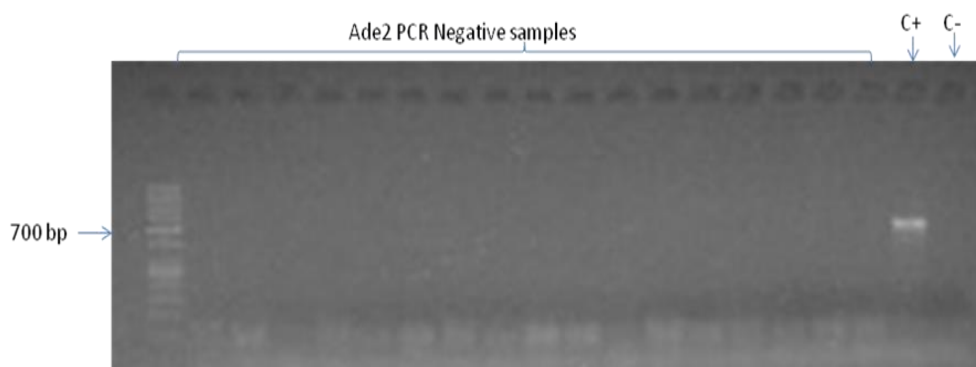

Figure 12: An example of gel electrophoresis of negative Ade2 PCR samples. The positive controls amplify the correct fragment size (≈ 700 bp).

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All the 18S PCR *T. congolense* savannah positive samples that were negative for the Ade2 PCR were submitted to this last PCR including the negative and positive controls in each. In all those PCRs the controls worked properly (Fig. 12).

Table 4: Distribution of proportions of the *Tco*AT1/*Tco*NT10 gene profiles in Matutuíne.

	***Tco*AT1/*Tco*NT10 gene profile**				
	Sensitive	**Resistant**	**Mixed**	**Total**	**%**
Bela Vista	21	23	36	80	55,9%
Zitundo	2	18	18	38	26,6%
Catuane	2	11	9	22	15,4%
Katembe	0	2	1	3	2,1%
Total	25	54	64	**143**	
%	17,5%	37,8%	44,8%		**100,0%**

For the 143 Ade2 positive samples, the *Dpn*II-PCR-RFLP was paerformed. The three possible profiles detected, were 17.5% (25/143) sensitive, 37.8% (54/143) resistant and 44.8% (64/143) mixed profile (Table 4). The mutation on the *TcoAT1* gene was detected in 126 of 143 samples from four of Matutuíne's evaluated administrative areas (Figure 13).

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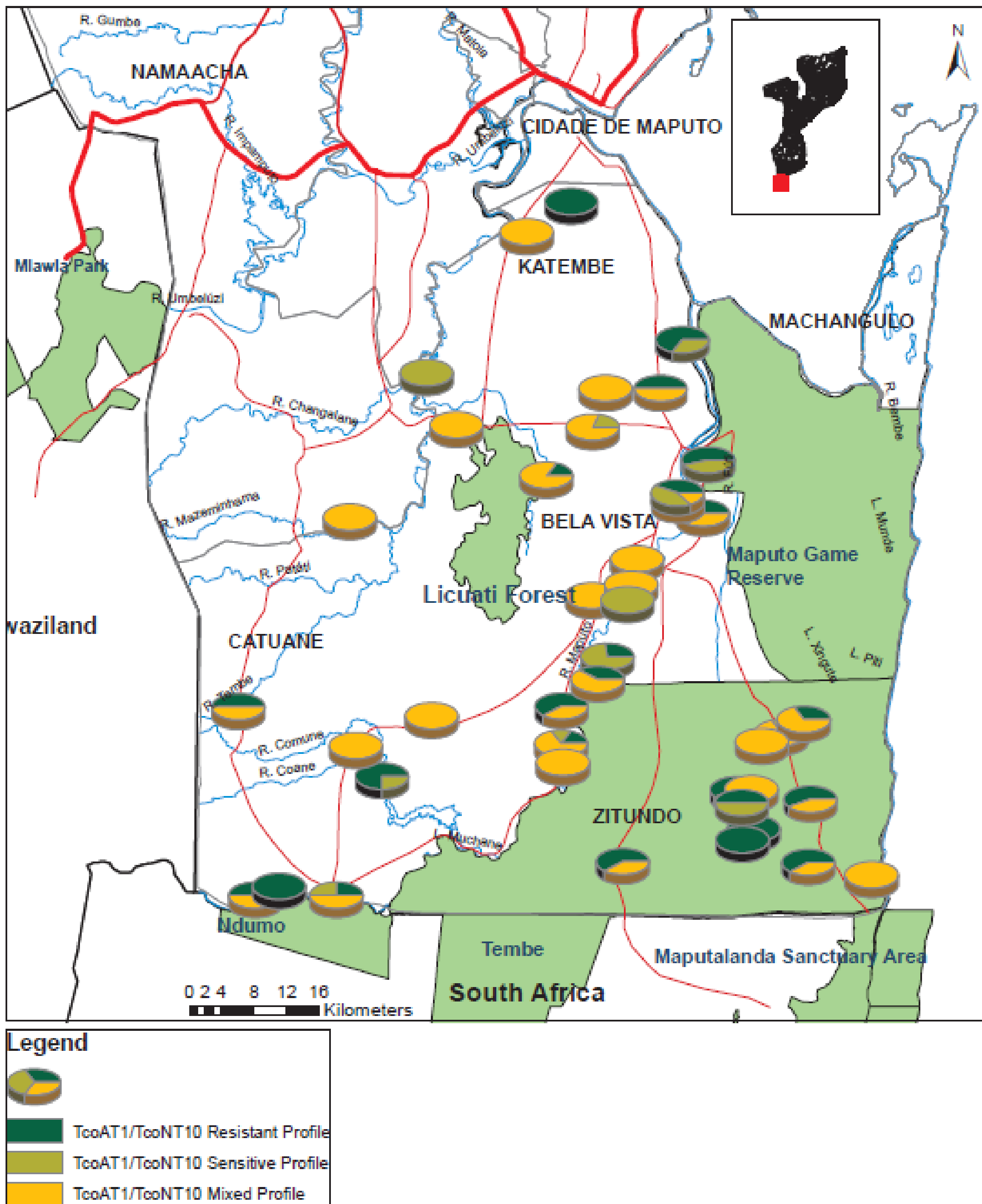

Figure 13: Distribution of the TcoAT1/TcoNT10 alleles in the Matutuíne administrative areas.

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3.4 The PCV variation between bovines infected by diminazene resistant or sensitive parasite

The mean Packed Cell Volume (PCV) of all sampled animals was 26.6%. The highest mean PCV (26.7%) was registered in Zitundo administrative area and the lowest (25.2%) in Catuane area. The PCVs were lower on the diminazene resistant parasite infected animals than in those of the drug sensitive parasite infected animals, 24.7% and 24.6% respectively, although the difference was not significant.

3.5 DA resistance: correlation of the molecular test with the mouse test

Of the 25 *Trypanosoma congolense* isolates collected, 24 were evaluated for DA resistance by both the *in vivo* mouse test and DpnII-PCR-RFLP. In the mouse test (24/24) were sensitive to the drug at the dose of 10 mg/kg and 20 mg/kg bw. In these assays, only the mice in the control group became parasiteamic during the observation period (2 months) and were used for the molecular evaluation of diminazene resistance status by DpnII-PCR-RFLP.

The molecular test revealed all three possible *TcoAT1* related profiles, with proportions of 5/24, 11/24, 8/24 of sensitive, resistant and mixed profile, respectively (Table 5). This indicated that 13/24 isolates had phenotype sensitive and 11/24 phenotype resistant (Table 6).

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Table 5: Mouse test and DpnII-PCR-RFLP results for the *T. congolense* isolates.

	10mg/kg DA		20 mg/kg DA		Non treated	
Strain	Mic.	DpnII-PCR-RFLP.	Mic.	DpnII-PCR-RFLP	Mic.	DpnII-PCR-RFLP
TIN MOZ 05	S (0/6)	ND	S (0/6)	ND	P (6/6)	S
ZIT Chit	S (0/6)	ND	S (0/6)	ND	P (6/6)	S
ZIT Chit 05	S (0/6)	ND	S (0/6)	ND	P (6/6)	S
TIN. Bot 05	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
TIN Bot 04	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
TCO Sa Mz1	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
ZIT Boa 06	S (0/6)	ND	S (0/6)	ND	P (6/6)	S
ZIT Chit 49	S (0/6)	ND	S (0/6)	ND	P (6/6)	S
JAN 2	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
MDA	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
MC 2	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
SAL	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
MC1	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
TC4 AD	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
ESP	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
J 261	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
TCBR	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
LF 19	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
MC6	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
MQ 26	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
DVAL	S (0/6)	ND	S (0/6)	ND	P (6/6)	R
J2	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
PBTIN 36	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
PBTIN 55	S (0/6)	ND	S (0/6)	ND	P (6/6)	M
Proportions	24/24S		24/24S			5/24S; 8/24M; 11/24R

S= sensitive; R=resistant; M=mixed; P=parasitemic; ND= none done

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Table 6: Concordance evaluation between the in vivo Mouse test and the DpnII-PCR-RFLP.

	DpnII-PCR-RFLP		
Mouse test (MT)	**Sensitive**	**Resistant**	**Total**
Sensitive	13	11	**24**
Resistant	0	0	**0**
Total	**13**	**11**	**24**

Kappa statistic calculation

Pr (a) : observed percentage agreement between both tests

Pr (a) = (total sensitive in both tests + total resistant in both tests) / total tested

Pr (a) = (13 + 0) / 24 = 0.542

Pr (e) : probability of random agreement

Pr (e) = (the probability that randomly results “sensitive” in both testes) + (the probability that in both testes the results "resistant" randomly)

Pr (e) = (0 x 0.542) + (1 x 0.458) = 0.458

$$\kappa = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)},$$ **K** = (0.542 – 0.458) / (1 – 0.458) = **0.15**

The agreement between the mouse test and the DpnII-PCR-RFLP revealed a slight agreement (K= 0.15) based on standards for strength of agreement for the kappa coefficient (≤ 0 poor, .01–.20 slight, .21–.40 fair, .41–.60 moderate, .61–.80 substantial, and .81–1 almost perfect).

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CHAPTER IV: DISCUSSION AND CONCLUSIONS

4.1 Discussion

4.1.1 Bovine Trypanosomosis status in Matutuíne

Matutuíne District has a long history of trypanosomosis, with the first case in bovines reported in 1908 and the first tsetse flies reported in the 1930s (Dos Santos Dias 1962). The region had been considered the most southern affected trypanosomosis area in Mozambique. In the present study, trypanosome infections were detected in bovines from all five sampled administrative areas. The parasitological technique reveals an overall prevalence of 17.3%. These prevalence are mostly comparable to the previous report in the same region 13 years ago, which was 13.8% (Sigauque *et al.* 2000). Thus, it indicates that the disease in the area is stable. Trypanosomosis in this region is efficiently controlled by chemotherapy, which seems to indicate that drug resistance is not a major issue in the area. Additionally, the observed high PCV values in bovines seem to indicate that chemotherapy is still effective to maintain a good health condition in the area.

The highest prevalence was found in Belavista (20.5% by parasitological test and 76.7% by molecular test) followed by Zitundo (16.6% and 33.5%, respectively) (Table 1). Belavista and Zitundo areas are connected to the Maputo Game Reserve. Consequently, these areas are closer to the potential trypanosome reservoirs, the wild animals. These two regions are the main administrative areas of Matutuíne, in terms of size as well as cattle population concentration.

Four species of trypanosomes have been reported in livestock in this southern trypanosomosis endemic region: the pathogenic *T. congolense*, *T. vivax, T. b. brucei* and the non-pathogenic *T. theileri* (Sigauque *et al.* 2000, Mamabolo *et al.* 2009, Gillingwater *et al.* 2010) in cattle and also *T. simiae* in pigs (Sigauque *et al.* 2000). The present results indicate that bovine trypanosomosis etiologic agents

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remain the same in Matutuíne with the detection of two *T. congolense* types (Savannah and Kilifi), *T. vivax,* and *T. brucei* (Table 2).

From the pathogenic trypanosomes, *T. congolense* is the major contributor for the general prevalence in Belavista, Zitundo Catuane and Katembe (Fig. 10). The incidence of *T. congolense* infections is higher in Belavista, (20.5% and 76.7% parasitologicaly and molecularly, respectively), followed by Zitundo (16.6% and 33.5%, respectively). A high prevalence of *T. congolense* has been reported in Matutuíne as well as in KwaZulu-Natal, South Africa (Gillingwater *et al.* 2010, Mamabolo *et al.* 2009). The regions are connected and are infested by the same *Glossina* species: *G. austeni* and *G. brevipalpis* (Sigauque *et al.* 2000, Gillingwater *et al.* 2010). *G. austeni* is described as a more competent specie in trypanosome transmission than *G. brevipalpis*, as it is speculated that is the main vector in this region (Motloang *et al.* 2012).

The prevalence of the non-pathogenic *T. theileri* infections was also higher by PCR than by parasitological technique (total of 16.2% against 1.2%, respectively). This can be explained by the very low parasitaemia observed with this particular species. The 18S PCR seems to be poorly sensitive for *T.* vivax detection (0.3% against 1.2% by parasitological test). Lower sensitivity for *T. vivax* detection by PCR tools based on rDNA amplification has been reported elsewhere (Constantine *et al.* 2005). The limited sensitivity of the 18S-PCR for this particular species is probably linked to a higher genetic diversity of the 18S locus. Additionally, microscopic observation of *T. vivax* is greatly facilitated by the high mobility of this specie.

The general discrepancy between the microscopic and molecular test results in this study (Table 1) can be related to the sensitivity of each diagnosis test. The molecular test is normally considered more sensitive and accurate than the microscopic test. Similar levels of discrepancies have been reported in previous studies (Mamabolo *et al.* 2009, Gillingwater *et al.* 2010), always in favour of the

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molecular test. However, the fact that 5.8% of the negatives in the 18S PCR test were positive in the parasitological test (Table 3) was intriguing. To try to overcome this constraint, the DNA of these samples was re-extracted from one of the spots on filter paper and re-evaluated by 18S PCR. All the samples remained negative. A whole genome amplification step was carried out with the REPLI-g® Ultra Fast® Mini kit (Quiagem®) in order to increase the DNA concentration of the samples, but, the samples remained negative on the 18S PCR test. A possible explanation could be a random effect linked to low parasitaemia. It is possible that the capillary tube examined by microscope contained by chance a trypanosome and that the capillaries used for spotting on filter paper contained no trypanosomes. This can be a consequence of the commonly low parasitaemia observed in the field.

4.1.2 Diminazene resistance in *T. congolense* strains circulating in bovines in Matutuíne

In Africa, it is becoming crucial to understand the dynamics of the TDR phenomenon in order to better overcome it’s potential impacts. Drug resistance in AAT is an old problem which is presently indicated as a bottleneck for disease control, and consequently for livestock livelihood in some trypanosomosis endemic regions, since chemotherapy is the only control system present in most of the endemic areas. Therefore, a great effort has been made in the last few years to improve the drug resistance diagnostic tools, with a notable contribution from molecular genetics (Delespaux *et al.* 2008a).

The diminazene resistance molecular diagnostic test, Ade2 PCR, revealed to be less sensitive than the species detection 18S PCR, since it was able to amplify only 21% (143 of the 689) of the *T. congolense* positive samples (Fig. 11).For all the negative samples, Ade2 PCR performeds correctly since the positive controls included in all PCRs always showed up (Fig. 12).Vitouley *et al.* (2012), also reported the low sensitivity of the Ade2 PCR (26%) when performed from field

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samples collected on filter paper, which normally presents a very low parasitemia, and consequently low target DNA concentrations. Additionally, the same authors reported that this PCR could be improved if an additional step of whole genome amplification on the first negative samples is added to the procedure. However, in the present study, the addition of the whole genome amplification step did not influence the final result. The gene targeted by the Ade2 PCR being a single copy while the 18S gene being a multicopy gene, may be the reason for this poorer sensitivity of the Ade2 PCR. However, notwithstanding the low number of Ade2 PCR amplifications, their amount was sufficiently representative to conduct a study on the present status of the Matutuíne District in terms of potential TDR occurrence, since only Machangulo administrative area was not represented. The large and representative initial study sampling (Fig 8) was crucially important to this fact.

According to Delespaux *et al.* (2008b), there are two genotypic characters for diminazene sensitive phenotype based on *Tco*AT1/TcoNT10 gene point mutation: the homozygous, with both alleles presenting the point mutation, and the heterozygous in which only one allele contains a mutation. The resistant phenotype is presented as a recessive homozygous genotype. The field distribution of the *Tco*AT1/*Tco*NT10 muted alleles is strongly related to the frequency usage of the diminazene aceturate trypanocidal drug (Delespaux and Koning 2013), suggesting that the study of the prevalence of the muted alleles may be used to classify regions where there are no relevant data about the frequency of drug usage, as is the case of Matutuíne District.

The present results indicate the occurrence in Matutuine of the three profiles of *Tco*AT1/*Tco*NT10 alleles: sensitive 17.5% (25/143), resistant 37.8% (54/143) and mixed (sensitive heterozygous) 44.8% (64/143). The*Tco*AT1/TcoNT10 allele's distribution in Matutuíne is more similar to that reported in regions without drug usage or with moderate drug usage than the distribution in regions where the drug usage frequency is higher (Table 7).

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Table 7: The *Tco*AT1 allele distribution in *T. congolense* isolates from regions with different drug usage frequencies. Adapted from Delespaux & Koning (2013).

Country	Area	Drug Usage	Number of *Tco*AT1 alleles muted (%) None	One	Both	Reference
Zambia, KwaZulu-Natal and Zimbabwe	National parks	None	3.0	61.8	35.3	(Chitanga *et al.* 2011)
Zambia	Eastern Province	Moderate	10.5	26.3	63.2	(Delespaux *et al.* 2008b)
Ethiopia	Ghibe Valley	High	2.7	2.7	94.6	(Moti *et al.* 2012)
Camaron	Adamaoua Plateau	High	0.0	0.0	100.0	(Mamoudou *et al.* 2008)

It might then be considered that Matutuíne is a region of moderate drug use for the control of trypanosomosis. This low level of drug use could be explained by the fact that the cattle breeding system in Matutuíne is mostly for subsistence with small farms and irregular usage of trypanocidal drugs. Conversely, in the Central region, where the cattle breeding system has been more commercial and based on intensive usage of trypanocidal drugs, the TDR phenomenon is well established (Jamal *et al.* 2005). The relatively high prevalence of the disease correlated with a low drug use is an indication that the *T. congolense* strains circulating in the area are lowly virulent. The same trend was observed during experimental infections (Neves *et al.*, unpublished data).

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4.1.3 Concordance between molecular and *in vivo* assays for *T. congolense* diminazene resistance diagnosis

In previous studies, the molecular test for *T. congolense* diminazene resistance diagnosis elaborated by Delespaux *et al.* (2006) correlated well with the mouse test; 60% concordance in Ethiopia (Moti *et al.* 2012) and Zambia (Delespaux *et al.* 2008b), 75% concordance in Cameroon (Mamoudou *et al.*, 2008), and 85.5 – 91.7% during the test validation (Delespaux *et al.* 2006).

From a total f 25 *T. congolense* isolates collected in Matutuíne, 24 were submitted to the mouse test and molecular test for diminazene resistance diagnosis. All isolates (24/24) were sensitive to diminazene aceturate by the in vivo mouse test at a dose of 10 and 20 mg/kg bw, since no parasites were found in any treated mice during the recommended 60 days of the test with a parasitological check twice per week (Eisler *et al.* 2001). However, the molecular test revealed 11/24 isolates with *Tco*AT1/*Tco*NT10 resistant profiles and 13/24 sensitive profiles (5 homozygous and 8 heterozygous) (Table 9). It seems that the molecular test is for the first time not in agreement with the mouse test. Chitanga *et al.* (2011), reported the lower sensitivity of the mouse test to detect resistant *T. congolense* strains when performed as described by Eisler *et al.* (2001) at 10 and 20 mg/kg, since he demonstrated numerous microscopically undetectable relapses during the testing period in mice infected with resistant strains, using the 18S PCR. This fact, does not compromise the mouse test itself, being actually a demonstration of the necessity of an improving the parasite-detection step.

The sensitivity of parasitological tests for trypanosome detection is recognized as being variable, since it can be strongly affected by many factors, including the blood volume of the sampled, the parasitaemia and even the skill and experience of the technician. PCR is now known to be more sensitive than microscopy for the detection of parasite but the frequency of observation is also a key factor. Chitanga *et al.* (2011) raised the observed relapse rate from 51% and 39% to 79.2% and

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66.7% (by PCR) for 10 and 20 mg/kg respectively with a simple increase in observation frequency from a single observation at the end of the observation period to 8 weekly observations. Exactly the same trend was observed in goats experimentally infected by *T. vivax* (Vitouley *et al.* 2012). The exact definition of a relapse should then be revisited.

Based on the present data (Table 4), the K statistic revealed a slight agreement between both tests (K= 0.15). Considering the present discordance between the two tests confirming part of the results of Chitanga *et al.* (2011), the following questions must be raised:

- If the mice were checked by PCR would they have been positive like in Chitanga's and Vitouley's experiments?
- What is the biological background of those very low persisting infections?
- Is the molecular test informative?
- Can we speak of drug resistance when an animal is relapsing but not showing clinical signs?
- What is the evolution of those very low parasitaemia: self cure or shift to a carrier state?

4.2 Conclusions

Looking at the results of this study we can conclude that in Matutuíne District:

- Trypanosomosis constitutes a minor threat in the Matutuine district;
- The resistant allele repartition indicates a low drug use in the area;

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- The in vivo drug sensitivity test in mice indicates no problem of diminazene resistance in the area, allowing the use of the sanative pair;
- The agreement between mouse test and molecular DpnII-PCR-RFLP for *T. congolense* diminazene resistance diagnose was slight (K = 0.15) when considering relapses detected by parasitological methods.

4.3 Recommendation

- The agreement between the two tests should be recalculated considering a detection of the relapses by molecular methods
- Further research is needed about the low fluctuating parasitaemias after treatment of an infected animal
- Considering the present results, the molecular tool for detection of resistance to DA should not be used at the individual level but rather considering the distribution of the resistance alleles as indicator of drug use intensity. This correlation should be further explored

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APPENDICES

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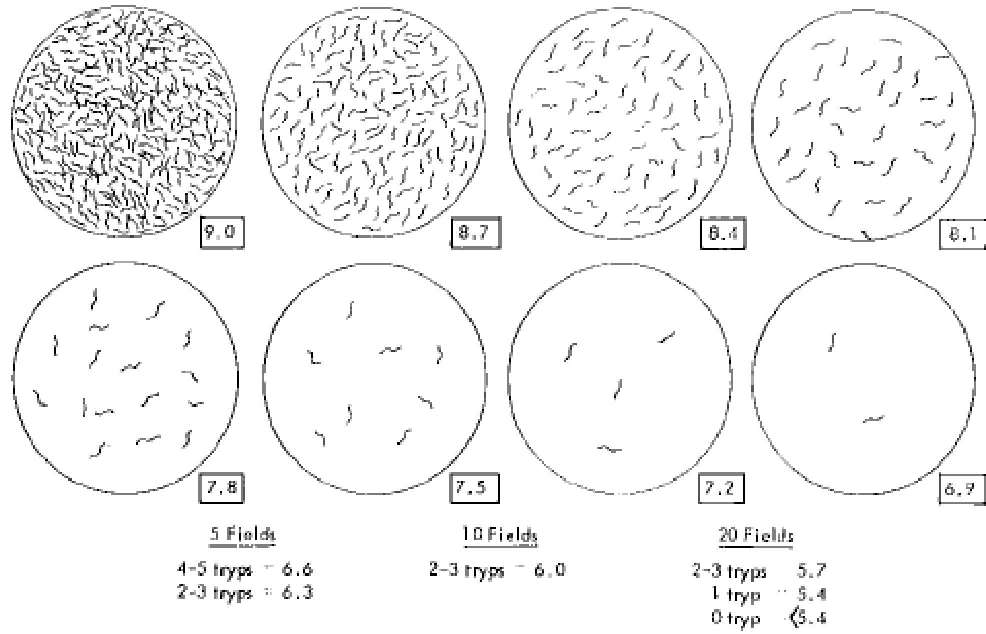
Appendix A: Herbert and Lumsden scale



Calculation

N° of trypanosomes /ml = $10^{\text{Herbert and Lumsden scale value}}$

Exemple: Herbert and Lumsden scale value = 9

N° of trypanosomes = 10^9 trypanosomes/ml

= 1 000 000 000 trypanosomes/ml

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Appendix B: Stabilate preparation from infected mice

1. ***Phosphate Saline Glucose (PSG)***

For 1L:			
	Na_2HPO_4	5.392	g/L
	NaH_2PO_4	0.239	g/L
	NaCl	1.7	g/L
	Glucose	10.0	g/L
	Ionic Strength	0.145	

Note that the glucose in PSG cannot be autoclaved. So the solution has to be autoclaved before the Glucose addition.

2. ***Dimethylsulphoxide 20% PSG preparation (DMSO 20% PSG)***

For 10 ml:

i. Add 8 ml sterile PSG to 2ml sterile DMSO

ii. Mix well the by vortex

iii. Make 1 ml aliquots (sterile glass flasks)

iv. Conserve at room temperature

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3. Procedures

i. Collect heart blood with a 2 ml syringe containing a tiny drop of heparin (5 µl max)

ii. Calculate the quantity of DMSO 20% PSG to add to blood: 3 parts blood for 1 part DMSO 20% PSG.

iii. Plan 0,4 ml per cryotube (if for example you collected 2,1 ml blood added to 0,7 ml DMSO 20% PSG this for a total volume of 2,8 ml, prepare 7 cryotubes).

iv. Label your cryotubes (Nalgen®) with strain code and date of stabilate preparation and align them with the graduations visible in front of you, unscrew all caps.

v. Mix in a tube the blood and the adequate volume of DMSO 20% PSG, invert the tube gently 10 times.

vi. With a plastic Pasteur pipette, dispense 0,4 ml of the mix in every cryotube.

vii. Place immediately in the insulated box in -80°C ultra-deep freezer for 24 hours then transfer cryotubes to liquid nitrogen. (If using the Nalgen® cryobox, replace isopropanol after every fourth time used).

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Appendix C : In vivo mouse test

1. Trypanosome retrieves and in vivo culture

i. To thaw a stabilate, remove it from the liquid nitrogen and place immediately in a water bath at 37°C.

ii. Inoculate two mice intraperitoneally with 0.1 to 0.3 ml of the undiluted thawed stabilate. Do not exceed 10 minutes between thawing and inoculation (cryopreservatives are toxic for the trypanosomes).

iii. Monitor the parasitaemia 2-3 times/week by examining wet films of tail blood. Clean scissors between mice.

iv. At a peak of parasitaemia or preferably at 7 on Lumsden scale, harvest three drops of blood (for a total of approx 10 µl) in 0,2 ml PSG solution.

v. Determine the parasitaemia using a Neubauer haemocytometer, if the quantities given above were correctly estimated it should be around 10^5 Tryps/0,2ml.

2. Diminazene aceturate (Berenil) dilution

i. Weigh 450 mg of Berenil powder and dilute in 10 ml sterile water (autoclaved or filtered) to get the stock solution. This can be stored at 4 – 8 °C for maximum 1 month.

ii. Dilute 1 ml of the stock solution in 9 ml sterile water (autoclaved or filtered) to get your working solution. Inject 0.5 ml/10g mouse or 0.1 ml/10g mouse for 10 mg/kg and 20 mg/kg groups, respectively.

This working solution should be discarded after group inoculation.

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3. Mouse test procedures

i. Mark 3 groups of 6 mice on the tail from 1 to 6 with black markers and take and record their individual weighs. During the experiment refresh the numbering before it fades out.

ii. Inoculate all 18 mice intraperitoneally with appropriately diluted parasitaemic blood (10^5 trypanosomes should be inoculated). If a problem happens during injection (injection under the skin or in the urinary bladder), indicate it on your record sheet.

iii. 24 hours after infection, treat the groups of 6 mice intraperitoneally with 0.1 ml/10g mouse of drug solution (in sterile water) to give the following doses:

Group 1 - Diminazene aceturate (Berenil) - 20 mg/kg b.w.

Group 2 - Diminazene aceturate (Berenil) - 10 mg/kg b.w.

Group 3 - Non-treatment control

iv. Following treatment, monitor wet films of tail blood twice weekly for 60 days for the presence of trypanosomes. Record your observations.

N.B.: The scissors used for this purpose should be cleaned in-between sampling each mouse. Remove all mice from the experiment (euthanasia with pentobarbital) the first day they are detected parasitaemic or wait that the parasitaemia reaches 8 or more on Lumsden scale and collect the blood for stabilate production and/or for DNA collection.

If all mice (or at least 5/6) in non-treated control group do not become parasitaemic, repeat experiment in new mice.

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4. <u>Results interpretation</u>

The results from the experiment will be interpreted on basis of the number of mice cured and the number treated in each group. If animals die without being detected parasitaemic the "number treated" for a group should be reduced accordingly.

Sensitive strain: 4 or 5 cured out of 5 or 5 or 6 cured out of 6.

Resistant strain: 4 or 5 relapses out of 5 or 5 or 6 relapses out of 6.

Intermediate: all other situations.

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Appendix D : Agarose gel electrophoresis

1. *2% Agarose gel preparation*

10 x TBE (Tris borato EDTA 10x)

0.89M Trizma	108g
0.89M Boric acid	55g
0.02M EDTA	7.44g
Double distilled water	up to 1 liter

Procedures

i. Prepare the gel casting devices for the horizontal electrophoresis as indicated by the manufacturer.

ii. Weigh the respective quantity of Agarose to reach 2% according to the final volume (e.g.: for 100 ml volume, 2 g agarose is needed).

iii. Up to final volume with 1x TBE, mix and oven it on microwave until it boils.

iv. Remove from microwave and let it cool (until it can be handled).

v. Add 5 µl of $5x10^{-4}$ g/ml Gel Red® per each 10 ml and mix well.

vi. Put the mix into the caster and leave the gels "polymerizing" for 20 min at room temperature.

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2. ***Loading the gel***

i. Remove the caster with the gel from the casting devices and place on the tank device (follow manufacturer instructions).

ii. Fill the tank with running buffer 1X TBE (follow manufacturer instructions for the quantity).

iii. Load in each gel wall, 5 µl of amplicon product sample mixed with 2 µl of 6x bromophenol blue loading buffer.

iv. Load also in each gel 4 µl per wall of 100 bp ruler (Fermentas®) for fragment size determination.

v. Cover the tank with the lid and connect the electrodes to a power supply and run the electrophoresis 100 V for 45 min.

vi. Take the gels picture under ultraviolet (UV) trans-illuminator.

3. Result interpretation

The samples will be considered positive if they present the expected amplicon fragment size, according with Geysen *et al.* (2003) for the species identification and Delespaux *et al.* (2008b) for the molecular drug resistance diagnosis

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Appendix E: Polyacrilamide gel electrophoresis (PAGE)

1. *Gel preparation*

Reagents for 2 mini gels

Reagentes	Stock concetration	Volume
Distilled water	-	9.45 ml
TBE	10x	1.6 ml
Acrilamida-Bisacrilamida	30%	5.5 ml
APS	25%	66.67 µl
TEMED	-	11 µl

Procedures

vii. Prepare the 1 mm thick gel casting devices for the vertical electrophoresis as indicated by the manufacturer.

viii. Mix all the reagents in thetable above, according to the sequence listed. Note that the gel polymerization will start just after addition of the two last reagents. So, ensure that you have all casting ready before adding those reagents.

ix. Put the mix between the two casting glasses for each gel and fit the combs for walls.

x. Leave the gels polymerizing for 2H at room temperature.

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2. Loading the gel

i. Remove the glass plates with the gels from the casting devices and place them on the tank device (follow manufacturer instructions).

ii. Fill the tank (inside and outside the gels) with running buffer 1X TBE (follow manufacturer instructions for the quantity).

iii. Load in each gel wall, 5 µl of restricted product sample mixed with 2 µl of 6x bromophenol blue loading buffer.

iv. Load also in each gel 3 µl per wall of 100 bp ruler (Fermentas®) for fragment size determination.

v. Cover the tank with the lid and connect the electrodes to a power supply and run the electrophoresis 20 V for 2.5 h at 18 °C.

3. Staining the gel

i. In a complete dark container (suitable for the gels size and amount) mix 50 µl of 1x TBE with 5 µl of 10 000X SYBER Green I® (Cambrex Bio Science Rockland, USA)

ii. Remove the gels from the glass plates and place them on the prepared SYBER Green I® (You must be able to differentiate the gels, for this purpose you can cut different corners)

iii. Cover the container with a dark lid and incubate at room temperature with medium horizontal shaking for 30 min.

iv. Wash the gels twice in sterile water and take the respective picture under the UV trans-illuminator.

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4. Results interpretation

The results of this procedure will be analysed according with Geysen *et al.*, (2003) for the species identification and Delespaux *et al.* (2008b) for the molecular drug resistance diagnose.

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