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ORIGINAL PAPER

Resistance to trypanocidal drugs in cattle populations of Zambezia Province, Mozambique

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Abstract

African animal trypanosomosis is a debilitating tsetse-transmitted parasitic disease of sub-Saharan Africa. Therapeutic and prophylactic drugs were introduced more than 50 years ago, and drug resistance is increasingly reported. In a cross-sectional study, 467 cattle were microscopically screened for trypanosomes. Samples were collected in May–July 2014 from five villages (Botao, Mungama, Zalala-Electrosul, Zalala-Madal, and Namitangurine) in Nicoadala district, Zambezia province. To evaluate treatment efficacy, trypanosome-positive animals in each village were randomly assigned to two groups, one treated with 0.5 mg/kg b.w. isometamidium (Inomidium®), the second with 3.5 mg/kg b.w. diminazene (Inomazene®). Cattle were microscopically monitored at days 0, 14, and 28 post-treatment. At day 28, trypanocides were swapped to investigate single or multiple resistance. Microscopically negative samples from the monitoring days were tested using 18S-PCR-RFLP. 22.9% (107/467) was found positive on day 0. On day 14, nine animals in Botao and seven in Mungama were positive. On day 28, in Botao, four animals from the diminazene group and four from the isometamidium group were positive. In Mungama, four animals from the diminazene group were positive on day 28. On day 42, six animals (9%) in Botao and two (9.5%) in Mungama remained positive after drug swap. No relapses occurred in Namitangurine. The 18S-PCR-RFLP consistently detected more positive than microscopy: indeed, positives reached 12, 13, and 8 in Botao and 9, 7, and 4 in Mungama, at days 14, 28, and 42, respectively. Single- and multi-drug resistance in Nicoadala district, Zambezia province, is thus here confirmed. This should be considered when choosing control options.

Keywords African animal trypanosomosis · Chemo-resistance · Trypanocides · Block treatment · PCR-RFLP

Introduction

African animal trypanosomosis (AAT) is a tsetse-transmitted, debilitating parasitic disease affecting both domestic and wild vertebrates (Barret et al. 2003). This disease is caused by protozoa, belonging to the genus *Trypanosoma* (Shah-Fischer & Say, 1981). In domestic animals, trypanosomosis is a disease with a great economic impact, affecting not only the well-being of the livestock population, but also efficient food production in crop-livestock production systems (Davila et al. 2003; Shaw et al. 2014). Among all African pathogenic trypanosome species, *Trypanosoma congolense* is arguably the one causing major losses in southern Africa (Sigauque et al. 2000; Sinyangwe et al. 2004; Specht 2008). The human form of the disease, known as sleeping sickness, is also sporadically detected in Mozambique, although disease surveillance is weak and under detection and underreporting are possible (Büscher et al. 2017; Franco et al. 2017).

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The treatment and prevention of trypanosomosis in cattle are carried out using three drugs, namely isometamidium chloride (ISM), diminazene aceturate (DA), and homidium bromide. In the last 50 years, no new trypanocides have been made available in cattle and drug resistance has been reported in several countries (Delespaux et al. 2008). In trypanosomosis, drug resistance is usually the result of the loss of capacity for drug uptake by the parasite, an alteration in drug-target interaction, or even a change in the efflux mechanism (Baker et al. 2013), as it is suggested for ISM (Sutherland et al. 1992).

Drug resistance is often associated to areas with high drug usage (Delespaux and De Koning 2007). This is compounded by the lack of new drugs and the incorrect utilization of existing ones (Geerts and Holmes 1998). Precise mechanisms of drug resistance are still undeciphered. However, in *T. brucei*, DA resistance was found to be associated with a mutation in a P2-type purine transporter, encoded by the *TbAT1* gene, which is responsible for the uptake of the drug by the parasite (Matovu et al. 2003; De Koning et al. 2004). In *T. congolense*, an orthologue gene of *TbAT1*, the *TcoAT1* gene, seemed to play the same role (Delespaux et al. 2006). However, Munday et al. (2013) found that for this particular species, *TcoAT1* acts in the transportation of inosine (P1-type purine transporter), as does the syntenic gene TbNT10 in *T. brucei*. The observed mutation in the *TcoAT1* gene does not change the DA sensitivity of the parasite but is associated to the phenotype as a genetic marker. The possible link between those two mutations remains unclear. Isometamidium kills protozoa by blocking the synthesis of nucleic acids. It intercalates between DNA base pairs and blocks the RNA and DNA polymerases. In sensitive strains, ISM is transported into and accumulated in the kinetoplast. It is assumed that changes in the efflux mechanism in the parasite result in a reduced accumulation of ISM inside the kinetoplast, thus creating resistance (Sutherland et al. 1991; Sutherland and Holmes 1993; Mulugeta et al. 1997).

In Mozambique, the use of chemotherapy for trypanosomosis control dates back to 1912 with the introduction of arsenic acid and later tartar emetic. Nevertheless, reports of failure of these compounds and, in some cases, the development of drug resistance were reported. In 1953, DA was introduced (Rafael 1959; Silva 1959). In the 1960s, ISM was introduced, and since then, it has been used, together with DA, as a chemoprophylactic and chemotherapeutic drug against trypanosomosis. Resistance to anti-trypanocidal drugs has been reported in at least 21 countries in Africa (Chitanga et al. 2011), including Mozambique, where it was experimentally confirmed for both ISM and DA (Jamal et al. 2005; Macucule 2014).

Despite the abovementioned explanations, the mechanism of resistance has not been fully elucidated for either drug. The lack of knowledge about the exact gene(s) that are responsible for the resistance profile in the livestock pathogenic species considered constrains the development of diagnostic molecular tools. In the absence of progress in this area, field tests (e.g., block treatment) can be considered as the alternative, as they have proven to be reliable, relatively easy, and fast to conduct. Furthermore, they do not require the isolation of trypanosomes (Eisler et al. 2001; McDermott et al. 2003). These advantages constitute the rationale of the approach used in the present study.

Drug resistance has been shown to be an important drawback to agricultural development in Africa in general, and in Mozambique in particular, where it has caused thousands of cattle deaths and a drastic reduction in the cattle population of Zambezia province. Despite this, local veterinary services have no structured program for trypanosomosis control and for regulating the use of trypanocidal drugs that is left to the initiative of the owner and/or animal health technicians. The present study provides an update on trypanocidal drug resistance in Nicoadala district and specifically evaluates the proportion of resistant strains in previously described hotspots. In addition to information on drug resistance, AAT epizootiology should be further investigated and the role of biological and mechanical transmission should be urgently revisited. Studies on vector species composition and distribution are in progress in order to shed light on the respective roles of tsetse flies and other biting flies in the transmission of trypanosomosis.

Material and methods

Study area

The study was conducted in five villages in Nicoadala district, Zambezia province (Fig. 1). The villages were chosen according to trypanosome prevalence obtained from a previous surveys conducted in Zambezia province between 2009 and 2013. The district has a history of high usage of trypanocides and has been previously identified as a drug resistance hot spot (Jamal et al. 2005).

Cross-sectional study and sampling framework

Blood samples for both parasitological and molecular analysis were collected in May–July 2014. Sample size was estimated according to Cannon and Roe (1982), where expected prevalence was 50% with a 95% confidence interval and a 5% desired absolute precision. In each of the villages, all animals were screened for trypanosomes using the buffy-coat technique (BCT) (Murray et al. 1977). Four milliliters of blood was collected using Vacutainer tubes containing di-sodium salt of ethylenediaminetetraacetic acid (EDTA) from the coccygeal vein of each animal. Two capillary tubes were then filled and sealed with crystaseal (Hawksley, Lancing, UK) and the blood was centrifuged at 1500 rpm for 5 min in a

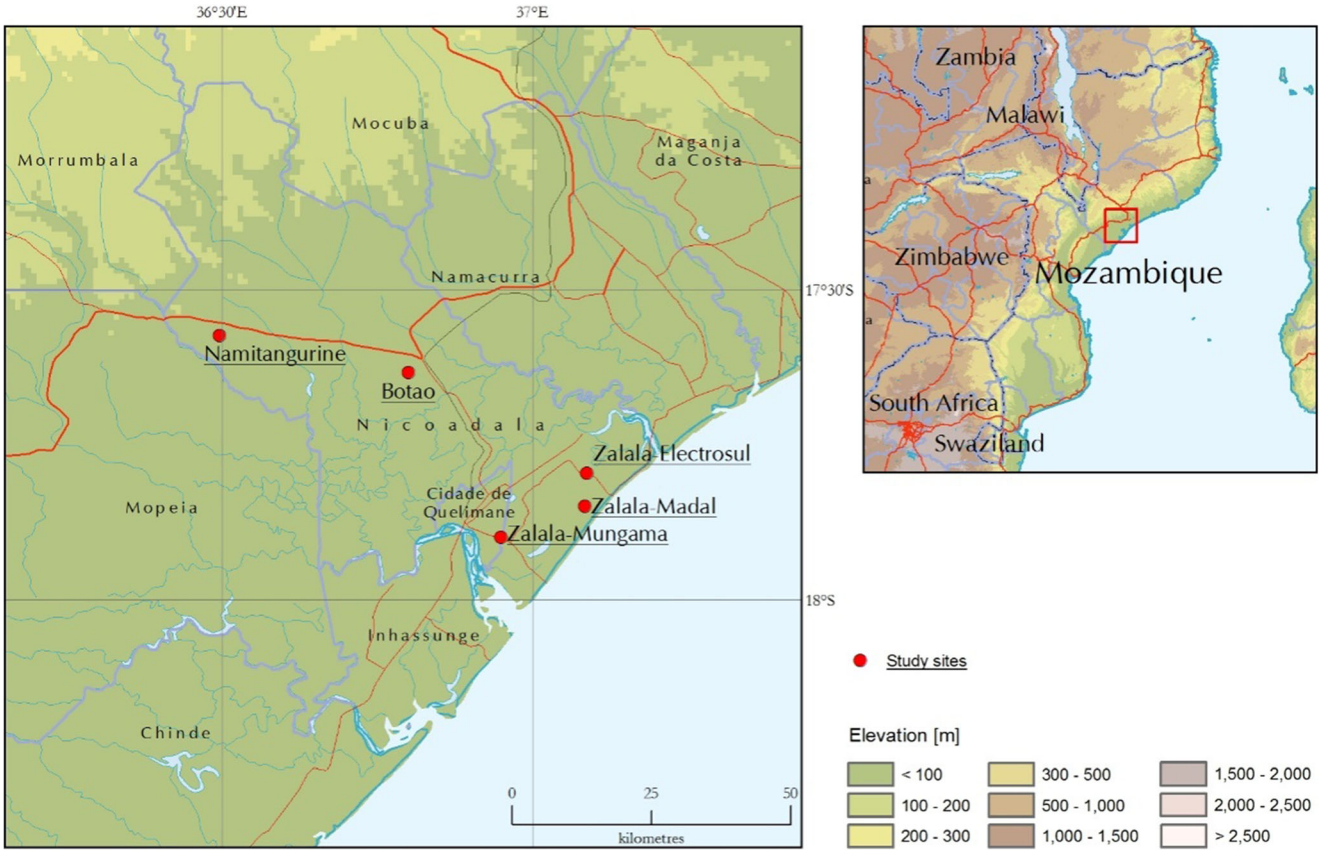

Fig. 1 The five study villages in Nicoadala district, central province of Zambezia. The study area is identified by the red square in the map of Mozambique

microhematocrit centrifuge (Hawksley®, Lancing, UK). One of the capillary tubes was used for packed cell volume (PCV) reading. The other was used for BCT and the preparation was examined for the presence of motile trypanosomes under a light microscope (Leica Mycrosystems, Wetzlar, Germany) at a magnification of × 400. In total, 467 heads of cattle from five villages were screened for trypanosomes.

Drug resistance study test

The trypanosome-infected animals from each village were randomly divided into two groups with a minimum of nine animals assigned to each group, for drug sensitivity tests of DA (Inomazene®, INOUKO Generics, Paris, France) and ISM (Inomidium®, INOUKO Generics, Paris, France). The number of animals per group was estimated according to the formula from Cannon and Roe (1982) to detect the presence of at least one resistant isolate, using an infinite population size and an expected proportion of trypanosome-resistant isolates of 30%. The first group was treated with 3.5 mg/kg of body weight (b.w.) DA and the second one with 0.5 mg/kg b.w. ISM (2% solution), as described by Mungube et al. (2012a). The animals were monitored for relapses on days 14 and 28 post-treatment, using the BCT method. At day 28, the trypanocidal drugs were switched to expose the strains previously treated with DA to ISM and vice versa, and to verify if there was resistance to only one drug (single-) or to both drugs (multi-resistance). Animals were monitored for 14 more days. At the end of the experiment, all animals that remained positive were treated with DA at 7.0 mg/kg b.w. All positive animals were ear-tagged. Animal ID, owner's name, location, GPS coordinates, age (calf, young, adult), sex, breed (zebu, taurine, crossbred), PCV, body condition (cachectic, lean, good), and infection type (trypanosome species) were recorded.

On monitoring days, for all animals, one vacutainer tube was filled with approximately 2 ml of blood per animal for DNA extraction and molecular tests (semi-nested 18S rRNA polymerase chain reaction (PCR)). Blood was kept at − 20 °C until analysis. Protocols and interpretation of the molecular analysis results followed the method described by Geysen et al. (2003).

DNA extraction and semi-nested 18S rRNA PCR

Genomic DNA was extracted from blood samples collected from the animals using a Qiagen® QIAamp DNA extraction kit (Qiagen, Hilden, Germany), following manufacturer

instructions. For the molecular detection of trypanosomes, 18S semi-nested rRNA PCRs were run, targeting a fragment of the 18S ribosomal RNA gene, on an Eppendorf Mastercycler® gradient thermocycler (Eppendorf AG, Hamburg, Germany), under the following conditions: 10 s at 98 °C; 40 cycles of 98 °C for 1 s, 58 °C for 5 s, 72 °C for 15 s and a final step of 72 °C for 1 min. For this, two reactions were carried out in a final volume of 25 μl containing 1× Phusion Flash high-fidelity PCR Master Mix (Thermo Fisher Scientific, Sweden), 200 μM of each dNTP, 20 pmol of each primer, and 5 μl of eluted DNA for the first reaction and 2.5 μl of PCR product from the first reaction for the nested reaction. Water was added to obtain a final volume of 25 μl. The primers used were 18ST nF2 5′-CAA CGA TGA CAC CCA TGA ATT GGG GA-3′ and 18ST nR3 5′-TGC GCG ACC AAT AAT TGC AAT AC-3′ for the first reaction and 18ST nF2 and 18ST nR2 5′-GTG TCT TGT TCT CAC TGA CAT TGT AGT G-3′ for the nested reaction. For PCR product visualization, the samples were run in a 2% agarose gel where 2 μl of loading dye was mixed with 5 μl of nested PCR product and loaded onto the gel. A 1000-bp DNA ladder was also loaded (4 μl) for fragment size determination, and the gel was run for 45 min at 120 V. The gel was stained with GelRed (Biotium, Inc., Fremont, CA, USA) at 4 μl per 100 ml of gel directly added to the gel before polymerization.

Restriction fragment length polymorphism

All the nested products (positive samples in agarose gel) were digested with *MspI* and *Eco571* (Acul) enzymes at 37 °C for 60 min, in a final volume of 15 μl containing 1× enzyme buffer, 0.5 μl *MspI*, 0.5 μl *Eco571*, 8.5 μl deionized water, and 4.0 μl of PCR product. The samples were then run in a 3% agarose gel at 80 V for 120 min, where 2 μl of loading dye was mixed with 4 μl of digested PCR product and loaded onto the gel together with a 100-bp DNA ladder. The gel was stained by adding GelRed (Biotium, Inc., Fremont, CA, USA) at 4 μl per 100 ml of gel before polymerization.

Results

Cross-sectional study: trypanosome prevalence

Out of the 467 animals screened using BCT, 107 were found to be positive for *T. congolense* infection in three of the five villages. No positive animals were diagnosed in both Zalala-Electrosul and Zalala-Madal villages. Only *T. congolense* infections were detected during the screening. Detailed description of the total prevalence per village can be found in Table 1. In the group of trypanosome-positive animals, the mean PCV (%) was 27%, significantly different from the negative sample group, i.e., 31% (P value < 0.0001, t test). The lowest PCV (12%) was found in one animal in Botao.

Treatment response to isometamidium chloride

On day 14 after treatment, three animals that had been treated with ISM were still positive in Botao, while in Mungama, four animals treated with ISM were positive. On day 28, the day on which the drug swap was carried out, four animals from the ISM group remained positive in Botao. In Mungama and Namitangurine however, no animals from the ISM group were found to be positive (Table 2).

Treatment response to diminazene aceturate

On day 14 after treatment with DA, the number of positive animals was six and three in Botao and Mungama, respectively. On day 28, four animals in Botao and four animals in Mungama were still positive after treatment with DA (Table 2). As was observed in the ISM group, no relapses were recorded in Namitangurine.

Multiple drug resistance

After swapping the drug, microscopic analysis on day 42 revealed six positive animals (9%) in Botao and two positive animals (9.5%) in Mungama, thus pointing to the presence of multiple drug resistance.

Molecular test

All the negative results from monitoring days 14, 28, and 42 were verified using semi-nested 18S PCR-RFLP. The 18S PCR allowed for the detection of positive cases where the BCT method detected negatives as shown in Table 2. A gel figure showing *T. congolense*-positive results detected by 18S PCR-RFLP can be found in Fig. 2.

Discussion

The Mozambican province of Zambezia is considered to be within an area infested by tsetse flies with medium to high trypanosome challenge, the “common fly belt” (RTTCP 2000).This is supported by the findings of the present study, where trypanosomes were detected in cattle in three out of five study villages. There may be several reasons for the absence of positive cases in Zalala-Madal and Zalala-Electrosul. One such reason could be the use of trypanocides, as it is known that most of the cattle in this area belong to the commercial sector and treatment with trypanocides is frequent (Specht 2008). However, the observed absence of positive cases could also be attributed to anthropogenic activities in the area

Table 1 Prevalence (by BCT) and mean PCV (%) in the five study villages in Nicoadala district (Mozambique), before treatment, May 2014

Village	Animals screened	Positive animals	Prevalence (%)	Mean PCV (%)
Botao	149	66	44.3	30
Zalala-Mungama	50	21	42.0	31
Namitangurine	120	20	16.7	28
Zalala-Madal	110	0	0	32
Zalala-Electrosul	38	0	0	31
Total/mean	467	107	22.9	31

(urbanization, clearing of vegetation for agriculture and new settlements), which may have altered the distribution of tsetse flies in the area or at least influenced their density. According to Malele et al. (2011), tsetse distribution can be affected by different factors such as changes in the land use and increases in human population and activities. These activities usually result in changes in the ecology of the areas previously occupied by tsetse flies, mainly through the elimination of breeding sites and hosts.

Specht (2008), working in Zambezia province in 2004, found a 15% prevalence of *T. congolense*, making it the dominant trypanosome species in the area. In the present study, only *T. congolense* infections were detected. Similar results were found by Jamal et al. (2005), when studying the susceptibility of *T. congolense* isolates in Zambezia province. In several studies conducted in sub-Saharan Africa, *T. congolense* has been found to be the most prevalent trypanosome species in cattle, especially in southern Africa (Sigaúque et al. 2000; Van Den Bossche 2001; Simukoko et al. 2007; Laohasinnarong et al. 2011; Mwandiringana et al. 2012; Simo et al. 2015), while in other areas, *T. vivax* is the dominant species (Ahmed et al. 2016; Cecchi et al. 2014).

One of the clinical evidences of trypanosomosis in cattle, especially in cases where it is caused by *T. congolense* infections, is low PCV (Mbewe et al. 2015; Marcotty et al. 2008). When comparing the mean PCV between trypanosome-negative and trypanosome-positive animals, a significant difference ($P < 0.0001$) was observed, with lower mean PCVs in trypanosome-positive animals.

Drug resistance (DR) to trypanocides is a dynamic process, and it is spreading and increasing in Africa. The detection of DR and identification of resistant *Trypanosoma* sp. populations remain a challenge. In the present study, a block treatment test was applied to assess single and multiple drug resistance to ISM and DA (Mungube et al. 2012a). PCR and BCT, which are both methods that are typically employed to estimate the prevalence of trypanosome infection, were used to detect ISM and DA treatment failure in the current study. Although BCT is a commonly used method for field assessment of trypanosomosis and for individual animal treatment monitoring, it is limited by the levels of parasitemia in the infected animals (Paris et al. 1982). The visualization of parasites in body fluids is currently the most widely used method for the diagnosis of animal trypanosomosis in endemic regions (Cecchi et al. 2014). However, according to Van den Bossche (2001), parasitemia can fluctuate below the levels of detection by microscopy. Nevertheless, with the advent of PCR, this limitation was surpassed, as it allows for the detection of trypanosomes in samples with very low parasitemia (Clausen et al. 1998; Desquenes and Davila 2002). Nonetheless, due to its high cost, PCR is not the technique of choice to support decisions on treatment options for individually diagnosed animals (Cox et al. 2010). In the present study, using

Table 2 Treatment response results, indicated as the number of animals remaining trypanosome-positive after treatment, verified by BCT and if negative checked by PCR-RFLP, in Zambezia Province in 2014

Village/method	Day 14		Day 28		Day 42	
	DA (%)	ISM (%)	DA (%)	ISM (%)	DA/ISM (%)	ISM/DA (%)
Botao/BCT	6/31 (19.4)	3/35 (8.6)	4/31 (12.9)	4/35 (11.4)	2/31 (6.5)	4/35 (11.4)
Mungama/BCT	3/10 (30.0)	4/11 (36.4)	4/10 (4.0)	0/11 (0)	2/10 (20.0)	0/11 (0)
Namitangurine/BCT	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Botao/BCT + PCR-RFLP	7/31 (22.6)	5/35 (14.3)	8/31 (25.8)	5/35 (14.3)	4/31 (12.9)	4/35 (11.4)
Mungama/BCT + PCR-RFLP	5/10 (50.0)	4/11 (36.4)	5/10 (50)	2/11 (18.2)	3/10 (30.0)	1/11 (9.0)
Namitangurine/BCT + PCR-RFLP	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)

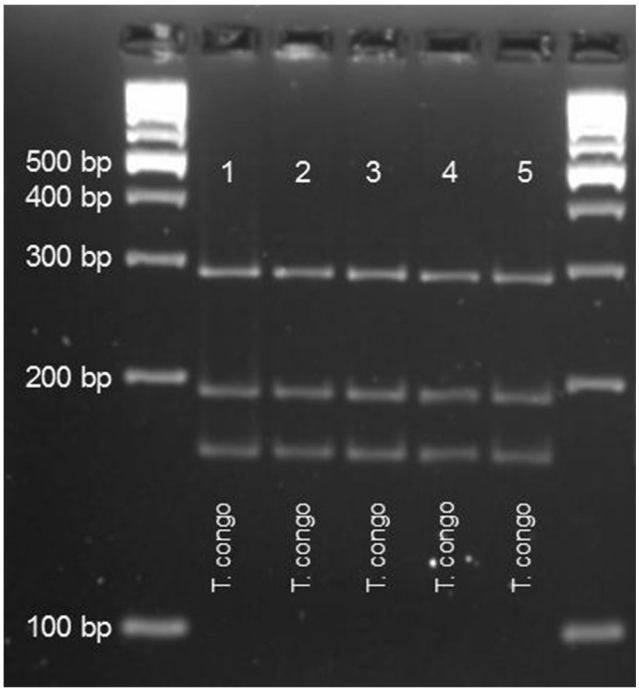

Fig. 2 RFLP restriction enzyme analysis using *MspI* and *Eco571* digestion of 18 Ssu-rDNA from *Trypanosoma congolense* isolates in agarose gel. One hundred-base pair DNA ladder was included in both sides on the gel

BCT, trypanosome-positive animals were diagnosed, treated, and monitored for relapses in the field. However, PCR was shown to be a more sensitive technique for the assessment of relapses as it allowed for the detection of positives in cases where BCT indicated negatives during the monitoring phase on days 14, 28, and 42 post-treatment.

Using the BCT method to check for trypanosomes 28 days post-treatment, the treatment failure rate for ISM was 6 and 0%, while the one for DA, it was 6 and 19%, in Botao and Mungama, respectively. These results revealed the difficulty that exists for farmers to be successful in the treatment of trypanosomosis. After the drug swap, conducted to detect multi-resistant strains, a total of eight isolates that were resistant to both drugs were identified on day 42. The present results confirmed those obtained by Jamal et al. (2005), which indicated that resistance to both ISM and DA is present in Zambezia province. Multi-resistant cases have also been detected, e.g., in Burkina Faso (Clausen et al. 1992), Zambia (Sinyangwe et al. 2004), Ethiopia (Miruk et al. 2008), Mali (Mungube et al. 2012a), and Togo (Tchamdja et al. 2017). In the present study, contrary to what was reported by Geerts et al. (2001), Mungube et al. (2012a), and Tchamdja et al. (2017), treatment failure was higher for DA than that for ISM. Multiple drug resistance can be a serious problem to cattle keepers, especially in this specific case, where the tested drugs are considered to be the most reliable ones presently available on the market.

Most of the cattle in Zambezia province belong to commercial farmers, and this is usually associated with intensive use of trypanocides. Although it cannot be assumed that the results from the present study are a reflection of this, it is a hypothesis that cannot be disregarded.

The results from the present study confirmed the existence of single- and multi-drug resistance in Nicoadala district, Zambezia province. Furthermore, based on the previous reports of resistance in the area, it is worth noting that the current results demonstrated that the geographical location of trypanocides-resistant hot spots has remained unchanged for at least the past 12 years. This information is fundamental when considering the control of trypanosomosis in the area. A better understanding of the drug resistance in the area will allow to define evidence-based, adapted measures for the progressive control of AAT (Diall et al. 2017).

The use of best bet strategies (Mungube et al. 2012b; Tchamdja et al. 2017) for the control of the disease in the area could be a valuable approach as it can be seen that the sanative pair of drugs can, at any moment, no longer be an option. The use of strategies involving rational drug use, which consists in the treatment of sick animals only and after proper diagnosis and using the correct dose should be urgently implemented by the local veterinary services. Moreover, general improvement of animal health conditions by deworming and reduction of animal disease risk, and vector control as described by Clausen et al. (2010) can also be of great impact. The development of molecular tools to allow for a faster assessment of the status of drug resistance is also advisable and should be encouraged.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and all procedures performed in studies involving animals were in accordance with the ethical standards of Biotechnology Centre—Eduardo Mondlane University and the practice at which the study was conducted. Permit number CBUEM/COMETH_0014/2014 issued on 17 March 2014.

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