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SHORT COMMUNICATIONS

Suspected resistance of *Trypanosoma* species to diminazene aceturate on a cattle farm in Nigeria

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Abstract

African animal trypanosomiasis is a major cause of mortality and economic losses for the livestock industry in Nigeria. Chemotherapy has been the most reliable option for cattle herders, and the most commonly found drug on the market is diminazene aceturate. To ascertain the long-term efficacy of this compound, we sampled a cattle herd in Ogun State, Nigeria, 2 months after they were treated with diminazene aceturate. The ITS-PCR results revealed 19 positives for trypanosome DNA out of the 79 samples tested (24.1%, 95% CI 16.0–34.5). Seventeen out of the total 19 positives were *Trypanosoma congolense* (21.5%, 95% CI 13.9–31.8). Mixed infections were also observed. Therefore, the persistence of bovine trypanosomiasis at this Nigerian cattle farm despite treatment could be due to diminazene aceturate resistant trypanosomes being present in the herd.

Keywords *Trypanosoma congolense* · Diminazene aceturate · Nigeria

Introduction

Bovine trypanosomiasis is a threat to Nigeria's livestock industry. *Trypanosoma congolense* and to a lesser extent *Trypanosoma brucei brucei* and *Trypanosoma vivax* have been widely reported to be the causal organisms of nagana in southwest Nigeria (Odeniran et al., 2019a). In Nigeria, nagana is often treated with diminazene aceturate; however, the persistence of the disease despite control measures is of major concern. The drug accumulates in the trypanosome mitochondrion and binds to the minor groove of DNA in a sequence-specific manner (Fox et al., 1990). The drug kills the trypanosomes by changing the topology of DNA and inhibiting topoisomerases, thus interfering with kinetoplast replication (Maser et al., 2003). Diminazene resistance appears to be less widespread than expected considering its intensive use over many decades (Geerts and Holmes, 1998; Delespaux and De Koning, 2007). Fake and substandard drugs (including trypanocides) are found at open drug markets (Kingsley, 2015) and the availability of these compounds may result in a greater risk of drug resistance developing. Recent studies on trypanosome drug resistance in Nigeria are lacking despite increased morbidity and mortality in the livestock industry (Sonibare et al. 2016). Therefore, in the current work, we analysed the presence of trypanosomes in cattle 2 months posttreatment with diminazene aceturate to identify resistant isolates and suggest improved chemotherapy options.

Methods

History

During a regional project on transmission dynamics of trypanosomes in cattle in southwest Nigeria, an incidental finding was observed. On April 16, 2016, cattle in the herd were diagnosed with trypanosomiasis by the farm manager, after which samples from the laboratory confirmed the presence of *Trypanosoma* spp. using microscopy. The trypanosome prevalence was estimated as 54.4% (95% CI 43.5–65.0) within a herd size of 79 cattle. The whole herd was immediately treated with diminazene aceturate (Nonazin®) at the recommended dosage of 3.5 mg/kg. At the farm, when cattle are

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examined for trypanosomes, high prevalence often leads to whole herd treatment with diminazene-based drugs. Nzi traps were set in the farm to investigate the vectors present on the farm during the study period (between April and June). Cattle on the farm were not treated with insecticides.

Sampling location and sample collection

Blood samples were collected on the 16th of June 2016 at an institutional farm in Abeokuta, Ogun State, during the rainy season (longitude 3° 25′ 9.14″ E and latitude 7° 13′ 58.59″ N). A total of 79 blood samples were collected on Whatmann® FTA (Flinders Technology Associates) filter paper cards and labelled appropriately. This represented 100% of the total herd. Cattle consisted of 73 White Fulani, four Sokoto Gudali, one Red Bororo and one N'dama cattle.

DNA extraction

DNA was extracted from punched Whatmann filter paper discs of dried blood samples using a blank to clean up after successive punching (Picozzi et al., 2002). The discs were washed twice with 1 mL of FTA purification reagent (Scientific Laboratory Supplies LTD, Nottingham, UK) for 15 min and removed. The discs were then washed to remove residual FTA purification reagent using 1 mL of 1 × TE (Tris-EDTA, Sigma-Aldrich Ltd. Gillingham, UK) solution; DNA was eluted with 5% (*w*/*v*) ultraviolet-treated Chelex-100 solution (AdventurerTM OHAUS, China) though being heated at 90 °C for 30 min in a Peltier thermal cycler (MJ Research Inc., USA).

DNA amplification

The ITS-rDNA primers forward 5′- CCG-GAA-GTT-CAC-CGA-TAT-TG -3′ and reverse 5′- TTG-CTG-CGT-TCT-TCA-ACG-AA -3′ primers (Njiru et al., 2005) were used with Mango-Taq™ master-mix. The mixture contains 5 × Mango-Taq™ buffer, 50 mM $MgCl_2$, 25 mM dNTPs, 5 U/μL Taq DNA and 12.6 distilled water. Following PCR, 10 μl of the reaction was expressed on 1.5% (*w*/*v*) agarose gel wells containing 10 μL GelRed™ nucleic acid stain (Biotium Inc., USA). Bands sizes were determined through comparison to a 100-bp (low range exACTGene™) molecular ladder (Fisher Scientific, UK).

Purification and sequencing

Bands were cut and purified using the QIAquick® gel extraction kit and quantified with nanodrop spectrophotometer, ND-1000 (Labtech, UK). All positive samples were sequenced unidirectionally for species identification (Eurofins GATC Biotech GmbH, Konstanz, Germany).

Data analysis

All analyses were done using WINPEPI (version 11.65) to analyse the general study prevalence of trypanosomes. The 95% confidence interval for descriptive analysis was calculated using the Wilson confidence interval. Significant differences were determined using Fisher's exact test, with $P < 0.05$ deemed to be significant.

Ethical approval

The study was conducted with the permission of the University of Ibadan Animal Ethics Committee (UI-ACUREC/App/12/2016/05) and in line with the guidelines of the committee.

Results

The prevalence of trypanosomes in the examined cattle herd through microscopy revealed 54.4% prevalence before treatment with diminazene aceturate. Based on visual determination under the microscope, the prevalence was estimated as 34.2% (95% CI 24.7–45.2) and 20.2% (95% CI 12.9–30.4) for *T. vivax* and *T. congolense* respectively.

Two months after treatment, the same set of animals was examined using molecular analysis. Of the 79 cattle tested, 19 were positive for trypanosome DNA (24.1%, 95% CI 16.0–34.5). The prevalence of trypanosomes prior and after treatment is shown in Fig. 1. All single infections after treatment revealed 14 positives of *Trypanosoma congolense* (17.7%, 95% CI 10.0–27.9) and one positive each for *T. vivax* (1.3%) and *T. brucei* (1.3%) complex, respectively. Mixed infections were two positives for *T. congolense* and *T. vivax*, and one positive for *T. congolense* and *T. brucei* at prevalence of 2.5% and 1.3% respectively. *T. congolense* showed a higher prevalence ($P < 0.0001$) when compared to the other species. Information on the cattle sex, cattle breed and trypanosome species are reported in Table 1.

The trapped flies only revealed the presence of stomoxyines at an apparent density of 107.5 and 35.7 flies/trap/day for *Stomoxys niger* and *Stomoxys calcitrans* respectively. There were no *Glossina* or tabanids in the trapped collections.

Discussion

The herd examined in this study showed DNA presence of trypanosomes 2 months after treatment with diminazene aceturate. This suggests that either the trypanosomes were resistant to diminazene aceturate or that the cattle were reinfected with trypanosomes. Although there might be some

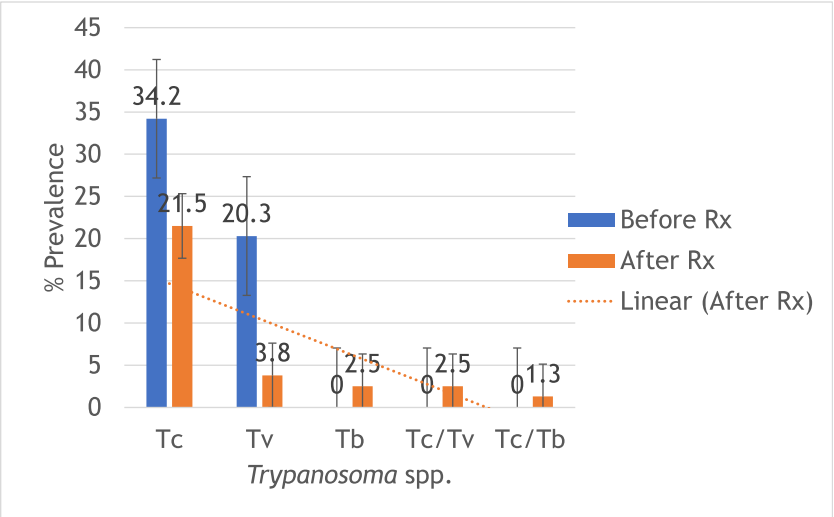

Fig. 1 Trypanosomes in cattle before (through microscopy) and after treatment (through molecular analysis). Tv *Trypanosoma vivax*, Tc *Trypanosoma congolense*, Tb *Trypanosoma brucei*, Rx treatment

reinfection, it would seem unlikely that 24.1% of animals would be DNA positive 2 months posttreatment and this may suggest that the trypanosomes were resistant. The presence of drug-resistant trypanosomes across Nigeria is not known, but there have been several complaints of ineffectiveness of diminazene aceturate in the field by cattle owners (Geerts and Holmes, 1998). Reinfection would require the presence of permissible vectors, and the cattle herders had complained about the abundance of *Stomoxys* spp. And this agreed with the trapped insects; however, tsetse flies and tabanids were absent from the trapped fly vectors. Hence, challenge from tsetse flies infected with trypanosomes seems unlikely. However, since the blood samples were collected 2 months after treatment with diminazene aceturate, there could be cases of reinfection from mechanical transmission from stomoxyines flies which were abundant in the area. Previous studies have shown that stomoxyine flies can transmit *T. congolense* (Sumba et al. 1998) and *T. vivax* (D'Amico et al., 1995) mechanically. Recently, Odeniran et al. (2019b) reported stomoxyines capacity for trypanosomes in southwest Nigeria. The presence of *T. brucei* after treatment is most likely due to the use of more sensitive molecular techniques.

The evolution of resistance to trypanocides will cause difficulties in the control and management of nagana. Similar problems are already observed in several African countries (Bengaly et al., 2018). Fake and substandard drugs in Nigeria could potentiate trypanocidal resistance resulting in major problems in the field (Kingsley, 2015).

Further studies need to be conducted in Nigeria to investigate if there is an increasing population of drug-resistant trypanosomes. Hence, there is a need to monitor infected animals treated with compounds for a long time involving several resampling and in vitro studies. Quality of trypanocides needs to be assessed to establish the compliance level of the drug constituents across the country. Strict regulations on drug importation, sale and administration should be instituted in Nigeria. Laboratory screening on all the trypanocides in the market will help to properly understand the control approach.

Table 1 Sex, breed and resistant isolates of trypanosomes of treated cattle

Variables	Appraisal of proportion and 95% confidence interval				
	T. congolense (Tc)	*T. vivax* (Tv)	*T. brucei* (Tb)	Tc/Tv	Tc/Tb
Sex					
Male ($n = 17$)	23.5 (9.6–47.3)	5.9 (0.7–35.8)	0.0	5.9 (0.7–35.8)	0.0
Female ($n = 62$)	21.0 (12.7–32.6)	3.2 (0.6–14.9)	3.2 (0.6–14.9)	1.6 (0.2–12.4)	1.6 (0.2–12.4)
Breed					
White Fulani ($n = 73$)	19.2 (10.1–33.4)	4.1 (1.0–14.8)	2.7 (0.5–12.8)	2.7 (0.5–12.8)	1.4 (0.2–10.7)
Sokoto Gudali ($n = 4$)	50.0 (10.5–89.5)	0.0	0.0	0.0	0.0
N'dama ($n = 1$)	100.0 (13.1–100.0)	0.0	0.0	0.0	0.0
Red Bororo ($n = 1$)	0.0	0.0	0.0	0.0	0.0
Sequences	U22319 (96–99%; $n = 12$), AB742531 (98–99%; $n = 5$)	KM391828 (98%; $n = 1$)	–	–	–

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval The study was conducted with the permission of the University of Ibadan Animal Ethics Committee (UI-ACUREC/App/12/2016/05) and in line with the guidelines of the committee.

Abbreviations Chelex, is a chelating material used to purify other compounds via ion exchange. It has the ability to bind transition metal ions. It was used to elute DNA from the FTA cards.; DNA, deoxyribonucleic acid; dNTPs, deoxynucleotide triphosphates; ITS, internal transcribed spacer; Mango, Taq™ master-mix- is a trade name for a PCR buffer with "mango" coloration; PCR, polymerase chain reaction; rDNA, ribosomal deoxyribonucleic acid; Taq, *Thermus aquaticus* used for polymerase chain reaction

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