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RESPONSE OF THREE NIGERIAN BREEDS OF SHEEP EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA VIVAX* TO DIMINAZENE ACETURATE THERAPY

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ABSTRACT

The efficacy of diminazene aceturate in three Nigerian breeds of sheep [West African Dwarf (WAD), Yankassa and Ouda] experimentally infected with *Trypanosoma vivax* was studied. Five rams of each breed were administered 0.5 ml of goat blood containing 2.5×10^6 *T. vivax* per millilitre intravenously, while three rams of each breed served as uninfected controls. The treatment with diminazene aceturate was intramuscularly administered to the infected sheep, when their packed cell volume (PCV) fell to 15 %, at a dosage of 7 mg.kg^{-1} b. w. The parameters measured were parasitaemia, live weight gain and PCV. By 24 hours post treatment (pt.), no trypanosomes were detected by either the Haematocrit Concentration Technique (HCT) or the Polymerase Chain Reaction (PCR) in the blood of any of the treated sheep. However, a relapse of parasitaemia occurred 17 to 32 days pt. in 46.7 % of the treated rams and these were retreated with 14 mg.kg^{-1} b. w. diminazene aceturate. There were gradual increments in the live weight gain and the PCV of the treated rams until the resurgence of parasitaemia. Ouda had the highest cases of relapse (80 %), the least mean live weight gain and was the only breed in which mortality was recorded despite the treatment. In conclusion, diminazene aceturate administered at 7 mg.kg^{-1} b. w. cleared the trypanosomes in the blood of all the treated sheep within 24 hours and this was accompanied by the restoration of lost weight and the reversal of anaemia. However, the subsequent resurgence of parasitaemia indicated that a dosage of 7 mg.kg^{-1} b. w. was no longer reliable for complete elimination of trypanosomes from all the tissues of the host.

Key words: breeds; diminazene aceturate; sheep; therapy; *Trypanosoma vivax*

INTRODUCTION

Trypanosomiasis is a vector-borne haemoprotozoan disease that affects man and other animals. It is caused by *Trypanosoma* species which are commonly transmitted cyclically by tsetse flies (*Glossina* spp.) and at times mechanically by other biting flies [10, 25, 33]. African animal trypanosomiasis is caused primarily by: *Trypanosoma congolense* (*T. congolense Savannah, T. congolense Forest, T. congolense Kilifi*), *T. vivax* and *T. brucei. Trypanosoma vivax* is one of the most pathogenic species of trypanosomes affecting a wide host range including cattle, sheep, goats, horses and donkeys; and can be transmitted both cyclically and mechanically. The disease is characterized by: undulating fever, anorexia, anaemia, body weight loss, lymphadenopathy, growth retardation, abortion and reduced fertility [9, 12, 16, 31].

In Africa, trypanosomiasis is controlled principally by means of chemotherapeutics or chemoprophylactic agents [19]. Other control methods are: reducing or eliminating tsetse flies with traps or insecticides and by selection of trypanotolerant breeds [37]. In Nigeria, the available trypanocides include diminazene aceturate, isometamidium, and homidium; but most times diminazene aceturate and isometamidium are the drugs used extensively [8, 11, 34]. There are widespread reports of resistance to trypanocides, particularly diminazene aceturate in the treatment of animal trypanosomiasis and several authors have reported it in different animals such as calves, sheep, dogs and rats [1, 3, 13, 14]. Some authors have attributed the resistance to sub-standard drug preparations, under dosing of the drug and overuse of the same type of trypanocide [15, 27]. D e K o n i n g [7] and D e K o n i n g et al. [8] associated the resistance to diminazene aceturate with reduced uptake of the drug by the trypanosomes. In Nigeria, the most common breeds of sheep are the West African Dwarf (WAD) in the forest zone, Yankassa in the grasslands and Ouda in the sahel/arid region. They serve as valuable supplements to cattle as sources of animal protein towards food security of the nation. Attempts to develop vaccines against trypanosomiasis have not succeeded mainly due to the continuous change of the protective surface antigen of the parasites [4, 21]. Also, efforts to control the disease through elimination of the tsetse vectors have largely been abandoned in sub-Saharan Africa due to fund constraints. No new veterinary trypanocide has been developed for over five decades and there is a very slim probability of the production of new trypanocides in the near future. Furthermore, these challenges are compounded by injudicious administration of the available trypanocides by unqualified personnel, including the animal rearers themselves. Consequently, there are increasing number of reports of resistance of trypanosomes to the existing therapeutic agents [15, 27, 32].

There is a dearth of information on the susceptibility to drugs of trypanosomes in sheep in Nigeria. This study was undertaken to evaluate the therapeutic efficacy of diminazene aceturate at a dosage of 7 $mg.kg^{-1}$ in three Nigerian breeds of sheep experimentally infected with *T. vivax*, as judged by parasite clearance, and the effects of treatment on the live weight and (PCV) of the infected animals.

MATERIALS AND METHODS

Experimental Design

Eight rams each from West African Dwarf (WAD), Yankassa and Ouda were obtained from Akinyele sheep markets, Ibadan, Oyo State and Teaching and Research farm, Alabata, Ogun State, and were acclimatized for a period of 30 days. The animals were housed in a pen partitioned into six units and a tsetse fly trap was positioned in the holding yard. Five rams of each breed served as the infected group while three rams of each breed served as the uninfected control group. Each breed of infected and control was housed separately in a fly-proof house. The sheep were screened for endo- and ecto-parasites and treated accordingly with albendazole at a dose of 10 $mg.kg^{-1}$ b. w. and cypermethrin pour-on at 6 $mg.kg^{-1}$ b. w. An isolate of trypanosomes obtained from naturally infected cattle was found to be *Trypanosoma vivax* [by PCR using internal transcribed spacer (ITS) primers]. The result was positive for *T. vivax*. This was further confirmed using a specie specific primer set (ILO 1264 and ILO 1265) for *T. vivax*. A goat that screened negative for worms and trypanosomes was used for the final passaging. The parasitaemia was estimated using a rapid matching method as described by H e r b e r t et al. [20]. The blood was diluted with normal saline to bring the parasitaemia to 5×10^6 per ml of the blood. Each sheep was infected with 0.5 ml of goat blood (containing approximately 2.5×10^6 *Trypanosoma vivax* organisms) through the jugular vein.

The parasitaemia was confirmed by both the haematocrit centrifugation technique (HCT) [28] and the PCR.

Thereafter, the PCR products obtained from experimentally infected sheep with high *T. vivax* parasitaemia were sent for sequencing to the Core laboratory of Cornel University (NY, USA). Each animal was treated with a freshly prepared 7 % solution of diminazene aceturate (Nozomil® Kepro B.V., Holland) intramuscularly at the rate of 7 $mg.kg^{-1}$ b. w. when the PCV fell to 15 %. The PCV limit of 15 % before initiating treatment was chosen because M u r r a y et al. [29] had shown that it was at this PCV value or less that death might occur. Relapsed cases were treated with 14 $mg.kg^{-1}$ b. w. of diminazene aceturate (Nozomil®). Where needed, a third treatment was effected with isometamidium at the rate of 1 $mg.kg^{-1}$ b. w.

About 3 ml of blood was collected through the jugular vein into an EDTA bottle from each of the experimental animals twice weekly for 6 weeks following the treatment with diminazene aceturate. The parasitaemia, live weight gain and PCV were measured and documented.

Determination of live weight gain

The live weight of all the animals were measured twice weekly using a hanging scale.

Determination of PCV

Blood was drawn into heparinized capillary tubes, one end of the tubes was sealed with plasticine and the tubes were centrifuged at 10,000 g for 5 min and the PCV was measured with a microhaematocrit reader [24].

Estimation of parasite centrifugation method of W o o [40] was used as described by M u r r a y et al. [28]. Briefly, the area immediately above the buffy coat in the haematocrit capillary tube was examined directly under the microscope for the presence of trypanosomes at 400× magnification. Afterwards, the buffy coat and upper most layers of red blood cells of the capillary tube were extruded onto a microscopic slide and examined with a phase—contrast microscope at ×400 magnification [28] for the presence of trypanosomes.

Detection of trypanosomes by polymerase chain reaction (PCR)

The DNA was extracted from the blood using QuickgDNATM Mini-Prep (Zymo Research Corporation, Irvine, CA, USA) as described by the manufacturer. Briefly, 400 µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed and incubated at room temperature for 5—10 minutes. The mixture was transferred to a spin column in a collection tube and centrifuged at 10,000 g for 60 seconds after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. A volume of 200 µl of prewash buffer was added to the spin column and centrifuged at 10,000 g for 60 seconds, after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 g for 60 seconds. The soluble DNA was eluted into a clean 1.5 ml micro-centrifuge tube, incubated at room temperature for 2—5 minutes and centrifuged at 16,000 g for 30 seconds. The eluted DNA was stored at –20 °C until use.

The PCR amplification was performed in 20 µl final reaction volume containing the equivalent of 20 ng of genomic DNA 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1 unit of Taq DNA polymerase (Bioneer, Inc. Alamada, CA, USA). The generic primers set that target the internal transcribed spacer 1 (ITS 1) used was (CF: 5'-CCGGAAGTTCACCGATATTG-3' and BR: 5'-TGCTGCGTTCTTCAACGAA-3' [30]. The primer set used for *T. vivax* was ILO 1264 and ILO 1265 and its primer sequence 5'-3" was CAGCTCGCCGAAGGCCACTTGGCTGGG and TCGCTACCACAGTCGCAATCGTCGTCTCAAGG [26].

The reactions were placed in a MJ MINITM Personal Thermocycler model PTC-1148 (Biorad, Hercules, CA, USA). The reaction conditions were as follows: For ITS initial denaturation of the DNA at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 58 °C for 1 min and 72 °C for 1 min with the final extension at 72 °C for 10 min. For *T. vivax* initial denaturation at 94 °C for 4 minutes followed by 35 cycles of 94 °C for 4 minutes followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s followed by the final extension at 72 °C for 5 minutes.

The gel electrophoresis procedure; 10 µl of the PCR products were electrophoresed through 1 % agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid 1 mM EDTA) at 90 V for 80 minutes, along with 10 µl of GENEMate Quanti-Marker 100bp DNA ladder (BioExpress, Kaysville, UT, USA). The gels were stained with GelRed® Nucleic Acid stain (Phenix Research Products, Candler, NC, USA) at 5 $\mu l.100\ ml^{-1}$ of the agarose gel suspension. After electrophoresis, the PCR products were visualized on a UV transilluminator and were photographed using an Alphalmager HP System (Protein Simple, Santa, Clara, CA, USA).

The PCR products obtained were sent for partial sequencing to the Core laboratory of Cornell University using a forward primer. The obtained sequences were BLAST for homology search in GenBank.

Data Analysis

The data obtained were summarized as the means ± standard deviation and the groups were compared by one-way ANOVA.

RESULTS

Parasitaemia before treatment

Parasitaemia was confirmed in all infected sheep by both the HCT and PCR techniques before the commencement of treatment with diminazene aceturate. The sequencing of the isolate of *T. vivax* used in this study revealed 99 % homology with the *T. vivax* with accession numbers U43183 and L25129 in GenBank (Fig. 1).

The phylogenetic tree was inferred using an unweighted pair group method with arithmetic mean (UPGMA) algorithm, involving a bootstrap procedure with 1000 replicate and evolutionary distance adjusted using the Kimmura-2 parameter.

Duration of infection prior to and outcome of the treatment

The mean (and range) period before the PCV of *T. vivax* infected sheep declined to 15 % (and hence required therapy) were 25.0 ± 12.1 days (18—39 days), 28.3 ± 12.9 days (14—39 days), and 23.5 ± 7.1 days (18—34) for WAD, Yankassa and Ouda, respectively. All sheep treated with 7 $mg.kg^{-1}$ b. w. of diminazene aceturate recovered, except one Ouda sheep that died one-day pt. Relapses of infection occurred in seven of the remaining 14 sheep: four (80 %) in Ouda breed, two (40 %) in the WAD and one (20 %) in the Yankassa. The mean (range) days to relapse were 21.75 ± 7.09 (17—32), 26.0 ± 7.07 (21—31) and 21 ± 00 (21) for Ouda, WAD and Yankasa, respectively.

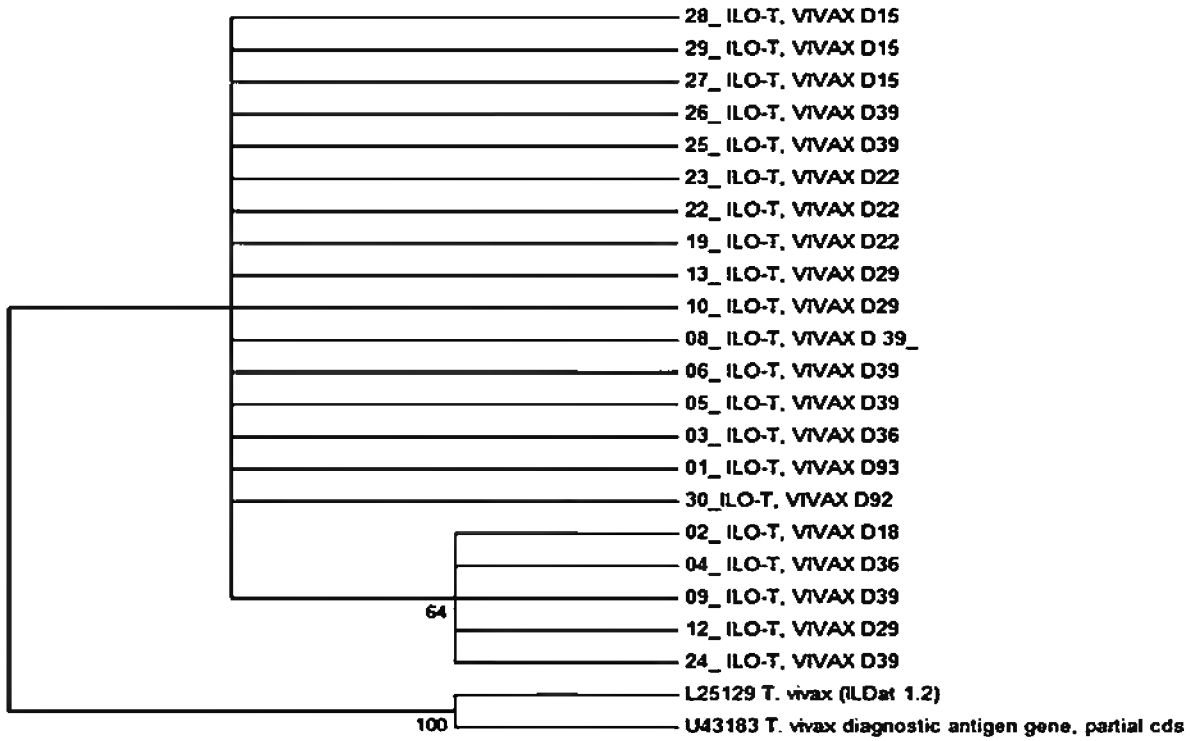

Fig. 1. Phylogenetic Tree of *T. vivax* isolates from this study (01_ILO-*T. vivax* to 30_ILO-*T. vivax*) and two *T. vivax* sequences obtained from GenBank

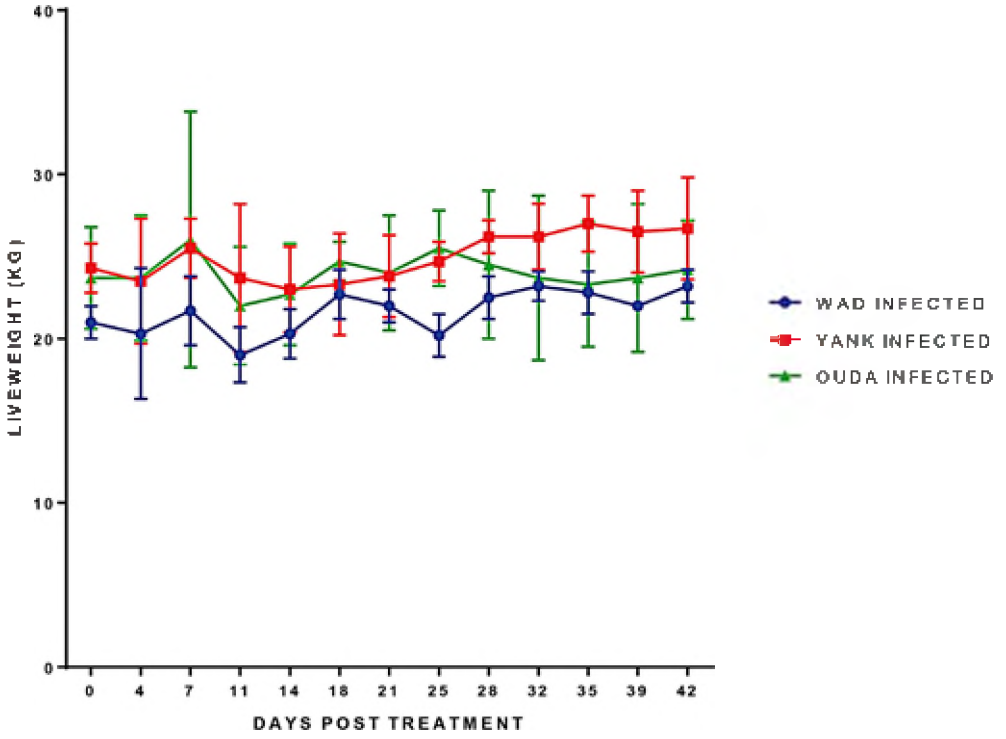

Fig. 2. Live weight values (means ± SD kg) of sheep following the treatment of experimental *T. vivax* infection with diminazene aceturate

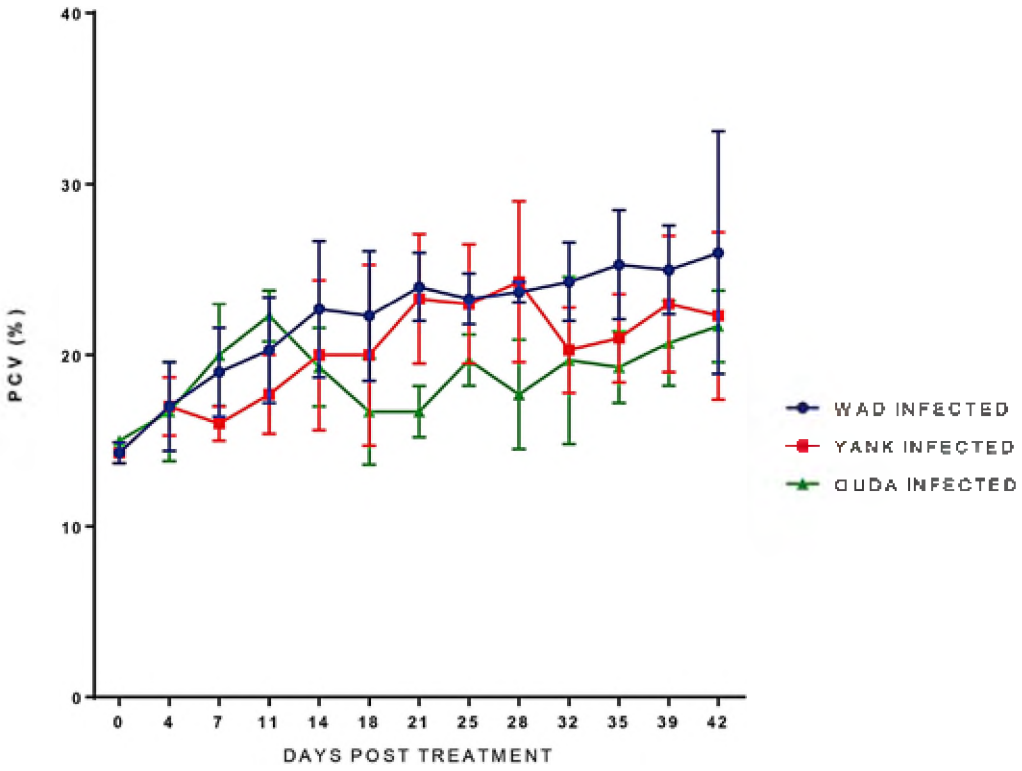

Fig. 3. Packed cell volume values (%; means ± SD) of the sheep following the treatment of experimental *T. vivax* infection with diminazene aceturate

These relapsed infections were re-treated with 14 mg.kg^{-1} diminazene aceturate. Of these, one sheep had a relapse of infection 27 days post second treatment and was treated with isometamidium (Trypamidium-samorinR) at a dose of 1 mg.kg^{-1}. The diminazene aceturate therapy study was finally terminated at day 42 pt. Throughout the period of the experiment, all of the uninfected controls were negative for trypanosomes.

Parasitaemia and live weight after treatment

By 24 hours pt., trypanosomes were not detected by HCT or PCR in the blood of any of the treated live sheep. The live weight of sheep after treatment with diminazene aceturate is presented in Fig. 2. The pre-treatment live weight of WAD, Yankassa and Ouda (21.0 ± 1.0, 24.3 ± 1.5, 23.7 ± 3.1, respectively) when compared with post-treatment live weight (23.2 ± 1.0, 26.7 ± 3.1, and 24.2 ± 3.0, respectively) at day 42 pt. were not significantly ($P > 0.05$) different.

Packed Cell Volume

The mean PCV of sheep before and after treatment with diminazene aceturate is presented in Fig. 3. In the WAD breed, there was a significant ($P < 0.001$) increase in the PCV from day 14 to day 42 while the significant ($P < 0.05$) increase in Yankassa breed was noticeable from day 21 to day 42. But in the Ouda breed, there was no significant increase in the PCV until day 42. There were significant ($P < 0.05$) increments when the pre-treatment PCV of WAD, Yankassa and Ouda (14.3 ± 0.6, 14.3 ± 0.6, 15.0 ± 0.0, respectively) were compared with post-treatment PCV (26.0 ± 7.1, 22.3 ± 4.9, and 21.7 ± 2.1, respectively) at day 42 pt.

DISCUSSION

The recommended dosage of diminazene aceturate for the treatment of trypanosomiasis in domestic animals is 3.5 to 7 mg.kg^{-1} b. w. [5, 18]. Due to the widespread twin problem of drug resistance and marketing of substandard drug preparations, the employment of higher dosages is necessary for effective therapy. This is especially so with severe disease that commonly follow experimental infections.

In this study, by 24 hours post treatment with diminazene aceturate at 7 mg.kg^{-1} b. w., there was disappearance of parasitaemia completely from the blood of the infected sheep (except for the one that died before the clearance of parasitaemia was checked at 24-hour pt.). This was confirmed by the HCT and PCR techniques which demonstrated the efficacy of the drug used. The subsequent relapse infections recorded in the various breeds (80 % in Ouda, 40 % in WAD and 20 % in Yankassa) may be an indication of the development of a level of resistance of the trypanosome isolates to diminazine aceturate. The relapse infections recorded in this study agree with the findings of B e n g a l y et al. [1] in which one sheep infected with *T. vivax* had a relapse infection after treatment with diminazene aceturate. Drug resistance to diminazene was also reported in *T. vivax* infected cattle [2] and in experimental *T. vivax* infected goats [38] in which relapse cases were recorded after treatment. The treatment of the infected sheep was not commenced immediately when the parasitaemia set in, but was delayed till the PCV declined to 15 %. This PCV limit of 15 % before initiating treatment was chosen because M u r r a y et al. [29] had shown that it was at this PCV value or less that the death of animals might occur and also to forestall unnecessary suffering of the experimental sheep. The set PCV limit of 15 % occurred at the mean of 25.0 ± 12.1 (18—39) days, 28.3 ± 12.9 (14—39) days, and 23.5 ± 7.1 (18—34) days post infection for WAD, Yankassa and Ouda, respectively. According to the reports of J e n n i n g s et al. [23] on *T. brucei* in mice and J a t a u et al. [22] on *T. evansi* in rats, the longer the duration of trypanosomiasis infection before treatment, the greater the chances of relapse. Therefore, the delay in the treatment of the sheep could have been responsible for the relapse infections that were recorded in this study. All infected sheep recovered except one Ouda, which died in spite of the chemotherapy in less than 24-hour post treatment. This might have resulted from an overwhelming of the host by the severe infection.

Following the treatment, the live weight gains and PCV of sheep showed a gradual increase until when parasitaemia reappeared in seven (7) of the remaining 14 infected sheep at different days post treatment (between days 17 and 32). This corroborated the report of E z e h et al. [14] that there was improvement in haematological parameters after treatment with diminazene aceturate. As a result of the incidence of relapses of the infections (Ouda, WAD and Yankassa had 80 %, 40 % and 20 %, respectively) at different days in the three breeds of sheep, the mean live

weight and mean PCV did not follow a particular trend (that is, they fluctuated). Ouda breed that had 80% relapse of infection had the least increase in live weight gain and PCV compared to WAD (40 %) and Yankassa (20 %) breeds. This suggests that, of the three sheep breeds, the Ouda was the most susceptible to the *T. vivax* infection.

Following re-treatment of the relapsed infection with 14 mg.kg^{-1} b. w. diminazene aceturate, no parasitaemia was seen again except in 1 Ouda sheep 27 days post second treatment. The relapses of the infections might have resulted from the localization of the trypanosomes in a site which the drugs could not reach. W h i t e l a w et al. [39] and O s ó r i o et al. [33] reported that in the aparasitaemic phase, *T. vivax* can be found in extravascular foci such as lymph nodes, the aqueous humour of eyes and cerebrospinal fluid; and these could serve as potential sources of the relapsed infections after the chemotherapy of trypanosomiasis. It is important to note that this study was not actually planned for a trypanosome drug resistance test in a ruminant model; therefore, graded dosages of diminazene aceturate and the required 100-day pt. duration for observation for complete recovery or relapse were not incorporated [6, 36]. However, the results of this study could serve as a preliminary test for further study on trypanosome drug resistance.

We also wish to point out that the *T. vivax* used for the experimental infection before treatment with diminazene aceturate was successfully passaged in mice and had 99% homology with those *T. vivax* sequence (accession numbers; U43183 and L25129) in the GenBank. There were very few similar reports of this rather unusual phenomenon which provides increased opportunity for experimental investigations with laboratory strains of *T. vivax*. *Trypanosoma vivax* is known to be difficult to cultivate in the laboratory and only a few *T. vivax* strains have been isolated that readily infect rodents. The most published *in vivo* work on this species comprises the very few mouse-infective strains; the main one being Y486 and its derivatives [17].

CONCLUSIONS

Diminazene aceturate administered at 7 mg.kg^{-1} b. w. cleared trypanosomes in the blood of all of the treated sheep in the three sheep breeds within 24 hours (except for one sheep that died in less than 24 hours pt.) and this was accompanied by restoration of lost weight and reversal of anaemia. However, the subsequent resurgence of parasitaemia indicated that a dosage of 7 mg.kg^{-1} b. w. is no longer reliable for complete elimination of trypanosomes from all of the tissues of the host.

From the results of this study, it can therefore be recommended that for a successful treatment without relapse using diminazene aceturate at a dosage of 7 mg.kg^{-1} b. w., the treatment should be carried out early enough before the PCV declined to 15 %.

In endemic environments, the treatment with diminazene aceturate at 7 mg.kg^{-1} b. w. should be followed up with isometamidium 1 mg.kg^{-1} two weeks after, to forestall the relapse or drug resistance as the latter have prophylactic properties. The toxicity of diminazene aceturate at 14 mg.kg^{-1} b. w. should be studied in sheep. New trypanocides should be developed to put an end to the problem of trypanosome drug resistance.

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