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African trypanosome infection patterns in cattle in a farm setting in Southern Ghana

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ABSTRACT

African Trypanosomiasis is a debilitating disease in both humans and animals that occurs in sub-Saharan Africa and has a severe negative impact on the livelihood of people in the affected areas. The disease is caused by protozoan parasites of the genus *Trypanosoma,* which is often described simply as blood-borne; however, a number of studies have shown the parasite inhabits many different environments within the host. Control of the disease involves measures that include the use of trypanocidal drugs to which there are growing number of reported cases of resistance. Here, the patterns of trypanosome DNA presence during a diminazene aceturate treatment round on a cohort of cattle in Adidome, Ghana were assessed. A group of 24 cows were selected irrespective of age and sex and the infecting trypanosome species followed for 18 days before and after treatment with diminazene aceturate in the blood and skin of the animals using a diagnostic nested PCR that targeted the alpha-beta tubulin gene array. Persistence of trypanosome DNA was observed over the period and parasite DNA was readily detected in both the skin and blood, with parasite DNA disappearing and reappearing in both across the study. Moreover, there was limited correlation between the parasite DNA detected in the skin and blood. Overall, the data show the patterns of a natural trypanosome infection during drug treatment. In addition, the diagnostic potential of sampling the skin for African trypanosomiasis is highlighted.

1. Introduction

African trypanosomiasis is a parasitic disease that affects both humans and animals and is caused by trypanosomes of the genus *Trypanosoma*. The human disease is caused by two subspecies of *T. brucei* - *T. brucei rhodesiense* and *T. brucei gambiense*, while Animal African trypanosomiasis (AAT) or nagana is caused by *T. congolense, T. vivax* and *T. brucei spp* (Bakari et al., 2017; Cecchi et al., 2009; Nakayima et al., 2012). African trypanosomes are routinely described as blood-borne parasites, but they infect many different environments within the host, including adipose tissue, skin, brain, spleen and liver (Silva Pereira et al., 2019; Tanowitz et al., 2017; Trindade et al., 2016). There is now a renewed appreciation of these sites of infection with more parasites thought to be present in the skin of humans than the blood (Camara et al., 2021; Capewell et al., 2016).

Even though the incidence of human African trypanosomiasis has reduced worldwide with less than 1000 cases reported in 2018, AAT still possess significant treat to sub-Saharan livestock (Buscher et al., 2017; Franco et al., 2020; N'Djetchi et al., 2017). This is a concern because studies have shown that animals are reservoirs of human-infective species (Hamill et al., 2013; N'Djetchi et al., 2017). It is estimated that about 55 million cattle in Africa are at risk of trypanosome infection (Cecchi and Mattioli, 2009) of which 3 million are thought to die every year (Giordani et al., 2016). AAT is also a serious developmental issue especially in areas of high endemicity. AAT impedes agricultural and economic development, as it is a major constraint of livestock production and causes a reduction in working capacity and efficiency of animals causing low crop production (Vreysen, 2006). This leads to poverty, hunger and poor nutrition in the affected areas and annual losses of between 1-4 billion US dollars to African farmers (Chanie et al., 2013; Swallow, 2000). Treatment of AAT is mainly by using diminazene aceturate or isometamedium chloride (Giordani et al., 2016; Steverding, 2008).

Like most countries in Africa, Ghana is also faced with the burden of

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trypanosomiasis, hindering productivity. In an earlier study at Adidome and Koforidua, it was found that the prevalence of trypanosomes in tsetse flies and cattle in the study areas were 17.4% and 57.5% respectively, with *T. congolense* the dominant species (Nakayima et al., 2012). While more recent studies focussing on Accra and Adidome identified *T. vivax* as the dominant pathogenic species (Bakari et al., 2017; Ofori et al., 2022). All these studies used primers that targeted either the internal transcribed spacer one (ITS1) of the ribosomal DNA or the tubulin gene array. The high prevalence of trypanosomes in the affected areas continues despite the use of control measures including chemotherapy and insecticides. Chemotherapy still remains the most effective option for controlling AAT and the widely used trypanocidal drugs include diminazene aceturate, isometamidium and homidium. These drugs were first used over 50 years ago and since then no new drugs have been developed (Giordani et al., 2016; Kroubi et al., 2011). Furthermore, the inadequate control of dosage, little control of drug sale and poor drug storage conditions has led to reports of resistance against these therapeutics (Delespaux and de Koning, 2007; Richards et al., 2021; Sinyangwe et al., 2004).

Given the expanding problem of resistance to trypanocidal drugs and the observation of continued trypanosome infection of cattle at the study site, the effect of a routine treatment of diminazene aceturate on trypanosome persistence in 24 cows within a herd was examined. Using a diagnostic PCR for trypanosomes, parasite DNA presence for 18 days after drug treatment in the blood and skin of the cows was followed. None of the cows showed a sustained loss of trypanosome DNA over this period, with parasite DNA readily detected in both the skin and blood. Furthermore, at the start of the study trypanosome DNA was detected in the skin of all 24 cows whereas in the blood DNA was only detected for 14 cows. Data from the current work highlight the changing patterns of trypanosome infection in a farm setting and the diagnostic potential of skin samples.

2. Material and methods

2.1. Ethical clearance

Ethical approval for this study was obtained from the Council for Scientific and Industrial Research (RPN002/CSIR-ACUC/2017). Permission was sought from the farm manager before the start of the research. The purpose, procedures, voluntariness, risks and benefits of the study were explained to the farm manager. All samples were collected by a trained veterinary technician.

2.2. Study area and cattle sample collection

The study was carried at the New Horizon farm located at Ameworlorkope, Adidome in the Central Tongu District, Volta Region, Ghana, which is ~130 km east of Accra. The site was selected due to the high prevalence of cattle trypanosomiasis (Bakari et al., 2017; Ofori et al., 2022). The total area of the farm is about 2 square miles and fenced. The cattle are Sanga breed, and the vegetation is semi forest and savannah. There are two dams in the farm from which the cattle drink. A stream passes through the farm but was dry during the period of sample collection. There is a crash pen where the cattle are treated, or samples taken for research, and two kraals where cattle are kept. The main trees in the area are neem and acacia.

Sanga cattle aged between 2-5 years irrespective of gender and breed were sampled. A total of 24 cattle were randomly selected and ear-tagged for the study (Table 1), as all cattle are routinely treated it was not possible to study untreated animals. Peripheral blood samples (5 mL) were collected by jugular venipuncture into S-monovette blood collection tubes with EDTA at 12 time points (before and after treatment (first sample taken before treatment on day 1 and subsequent samples taken on day 2, 3, 4, 5, 6, 7, 9, 11, 15, 17 and 19)) with survidim (diminazene aceturate at 3.5 mg/kg (w/w)) by the farm manager. The drug dose used was partly informed by earlier studies that reported the dominant presence of both *T. congolense* and *T. vivax* in the area (Bakari et al., 2017; Nakayima et al., 2012; Ofori et al., 2022). Blood smears were examined by microscopy, but no microscopy positive sample was identified and this maybe because of the low levels of parasitemia that is associated with AAT. Blood samples were transported on ice to the University of Ghana for DNA extraction. Skin biopsies (~5 mm diameter) from the back (between the hook and thurl) were also taken from the cattle using a clean razor blade for each biopsy, at 3 time points (1 before and 2 after treatment) in accordance with livestock welfare advice. Each skin sample was put in 200 μL absolute ethanol. A total of 280 EDTA blood samples and 71 skin biopsies were collected over the sampling period. For some time points, not all cows were sampled, as it was not possible to corral them.

Table 1
Characteristics of selected cattle at the study site.

Cow ID	Sex	Age (Y)	Estimated number of prior drug treatments	No. of times blood collected
AD 00523	F	3	6	12
AD 00524	M	4	8	12
AD 00532	M	4	8	12
AD 00539	M	3	6	12
AD 00541	F	5	10	12
AD 00546	F	5	10	11 (no day 7)
AD 00565	F	5	10	11 (no day 19)
AD 00566	F	2	4	12
AD 00570	F	5	10	12
AD 00573	M	3	6	12
AD 00575	M	4	8	12
AD 00585	M	2	4	12
AD 06448	F	4	8	11 (no day 15)
AD 06472	M	2	4	11 (no day 3)
AD 06811	M	3	6	12
AD 06813	M	3	6	12
AD 06814	M	2	4	12
AD 06815	M	2	4	12
AD 06817	F	2	4	11 (no day 17)
AD 06818	M	2	4	12
AD 06819	M	2	4	12
AD 06820	M	2	4	11 (no day 2, 5)
AD 08323	M	2	4	12
AD 08329	M	2	4	11 (no day 7)

2.3. Molecular identification of trypanosomes in skin biopsies and blood of cattle

Genomic DNA was extracted from blood using the Qiagen QIAmp DNA blood mini kit (Hilden, Germany). For the skin biopsies Qiagen

DNeasy tissue and blood kit (Hilden, Germany) was used for gDNA extractions. The extracted DNA samples were stored at -20°C. A sensitive and species-specific nested PCR was performed by targeting the intergenic region between the alpha and beta tubulin genes in the trypanosome genome (Ofori et al., 2022). All PCR amplifications were conducted in a total reaction mixture of 50 µL containing 5x Mango Taq buffer, 1.5 mM $MgCl_2$, 0.33 µM outer primers (forward and reverse) as shown in S1 Table, 0.33 mM dNTPs, 2.5 U Mango *Taq* polymerase and 2 µL gDNA as the template. The thermal cycling condition were 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 45 seconds, plus 3 seconds for each cycle, and a final extension of 72°C for 5 minute and then cooling at 4°C. The nested step was done using a reaction mixture of 50 µL made up of 2 µL of the first PCR products. The components of the second reaction and cycling conditions were the same as first the reaction except for the outer primers being replaced with inners primers designed based on the tubulin alpha-beta intergenic regions (S1 Table). Amplicons were resolved on a 1.5% (w/v) agarose gel and trypanosome identification was based on the length of the PCR product - *T. brucei* (424 bp), *T. congolense* (456 bp), *T. vivax* (586 bp) and *T. theileri* (646 bp). Gel extraction was performed for selected PCR products using the Qiagen QIAquick Gel Extraction Kit (Hilden, Germany). The purified DNA was then stored at -20°C before being sent for Sanger Sequencing. For the BLAST analysis, the BLAST interface available through the TriTrypDB website was used and the genome sequence of the relevant reference trypanosome species was searched using the default parameters. The analysis was done using the first 450 nucleotides of the sequence and used the top BLAST hit for species assignment (S2 Table).

3. Results

3.1. Trypanosome DNA persists after diminazene aceturate treatment

To understand the patterns of trypanosome infection during a treatment cycle, a randomly selected cohort of 24 cattle were followed before and after diminazene aceturate treatment. Blood samples were collected at day 1 (before treatment) and days 2, 3, 4, 5, 6, 7, 9, 11, 15, 17, and 19 (after treatment). Nested PCR was performed on the genomic DNA extracted from the blood to determine trypanosome DNA presence before and after treatment. The predominant species detected was *T. brucei*, with *T. congolense* and the non-pathogenic *T. theileri* also detected, but less frequently; however, *T. vivax* another species which causes AAT was not detected (Fig. 1 and S1 Fig, and S2 Table). For the majority of positive samples only one species of trypanosomes was detected but co-infections of *T. brucei* with *T. theileri* and *T. brucei* with *T. congolense* were seen (Fig. 1 and S2 Table).

The patterns of trypanosome DNA detection in the 24 cows across the time course were followed and there were cows in which the trypanosome species varied from day to day – for example AD 06472, AD 06819 and AD 06820. However, there was no clear evidence for reduction of parasite DNA below detectable levels after treatment. Three broad categories of pattern were identified (Fig. 1A-C). 1) Persistence of trypanosome DNA (8/24) - defined as the same trypanosome species appearing in at least four consecutive time points, with no more than three time points in total negative for trypanosome DNA after treatment (Fig. 1A). 2) Loss of trypanosome DNA, with a short gap before the DNA was detected again (5/24) - defined as the disappearance of same trypanosome species for two sequential time points after treatment with a subsequent reappearance of the parasite DNA (Fig. 1B). 3) Loss of trypanosome DNA, with a longer gap before the DNA was detected again (11/24) - defined as the disappearance of same trypanosome species for three or more sequential time points after treatment with a subsequent reappearance of the parasite DNA (Fig. 1C).

Given the variation of detection patterns within individual cows, the patterns of DNA detection at the cohort level were examined to determine whether any patterns were observable. The frequency at which cows tested positive for trypanosome DNA for each time point was determined (Fig. 1D). Trypanosome DNA was detected across all the time points, with no large changes in the number of cows infected across the course. Again, pointing to a lack of consistent clearance of trypanosome DNA below the detectable limit of PCR.

3.2. Trypanosome DNA was readily detected in cattle skin before and after treatment

Despite trypanosomes being described as blood-borne parasites, they were historically considered a tissue parasite and there is a long-standing literature showing them present in many different tissues, including the skin (Silva Pereira et al., 2019; Tanowitz et al., 2017; Trindade et al., 2016). Therefore, skin biopsies at selected time points were taken (Day 1, 6 and 19) to follow the effect of treatment on the detection of trypanosome DNA in the skin (S2 Fig). Before treatment, parasite DNA was found in all 24 cows, with one mixed infection (*T. brucei* and *T. congolense*) (Fig. 2 and S2 Fig). There was a slight drop in the detection of parasite DNA across the time course, with 17 of the 24 cows (70.8%) positive at day 6 and 15 of the 23 (65.2%) positive at day 19. However, the patterns were not clear-cut as only two of the seven cows that were negative for trypanosome DNA at day 6 were found to be negative at day 19 (AD 06817 and AD 00539) (S2 Fig).

3.3. Limited correlation in the presence of trypanosome DNA between the skin and blood

To further investigate the dynamics of trypanosome infection, the patterns of parasite DNA detection were compared in the skin and blood of cattle (Fig. 2). At day one before treatment, trypanosome DNA was detected in all skin biopsies (100%) while trypanosome DNA was detected in only 14 out of 24 blood samples (58.3%). At day six, 17/24 and 14/24 of the cows were positive for trypanosome DNA in the skin and blood by nested PCR respectively. However, only two cows were negative for trypanosome DNA in both the skin and the blood (AD 06817 and AD 00539). At day 19, 15/23 and 17/23 of the cows were positive for trypanosome DNA in the skin and the blood respectively, with only one cow negative for trypanosome DNA in both (AD 08323); however, at day 6 this cow had been positive for trypanosome DNA in both the blood and the skin. Furthermore, for the blood samples only two cows which were negative at day 19 were also negative at day 6 (AD 00566 and AD 06814). Overall, the species detected in the skin was likely to be observed in the blood as well but there were numerous exceptions. Finally, it was noted that there were eight occasions over the time course when the species detected in the blood differed from those in the skin. For example, at day 6 in the blood of cow AD 06472 *T. theileri* was detected, whereas *T. brucei* was found in the skin (Fig. 2).

To confirm the trypanosome species identified by nested PCR, 39 selected PCR products from the skin biopsies and cattle blood were Sanger sequenced and the identity to the reference genomes was determined. 26 samples had clear chromatograms, with the sequence matching the species predicted by PCR product size (S2 Table). 13 samples had chromatograms in which multiple traces were seen and for these samples eight matched the predicted species, while five did not (S2 Table).

3.4. Sex of cattle did not affect response to treatment

Several studies have shown that differences in the sex and age of cattle may influence the trypanosome infection rate (Ngongolo et al., 2019; Simwango et al., 2017). To determine whether the sex and age of the cattle affected the trypanosome DNA detection pattern, the percentage of time points for which a cow tested positive for trypanosome DNA in the blood was determined. Across the sampling period male cows were positive for trypanosome DNA for 60.4% of the time points and female cows 59.6%, indicating that sex likely had no effect on

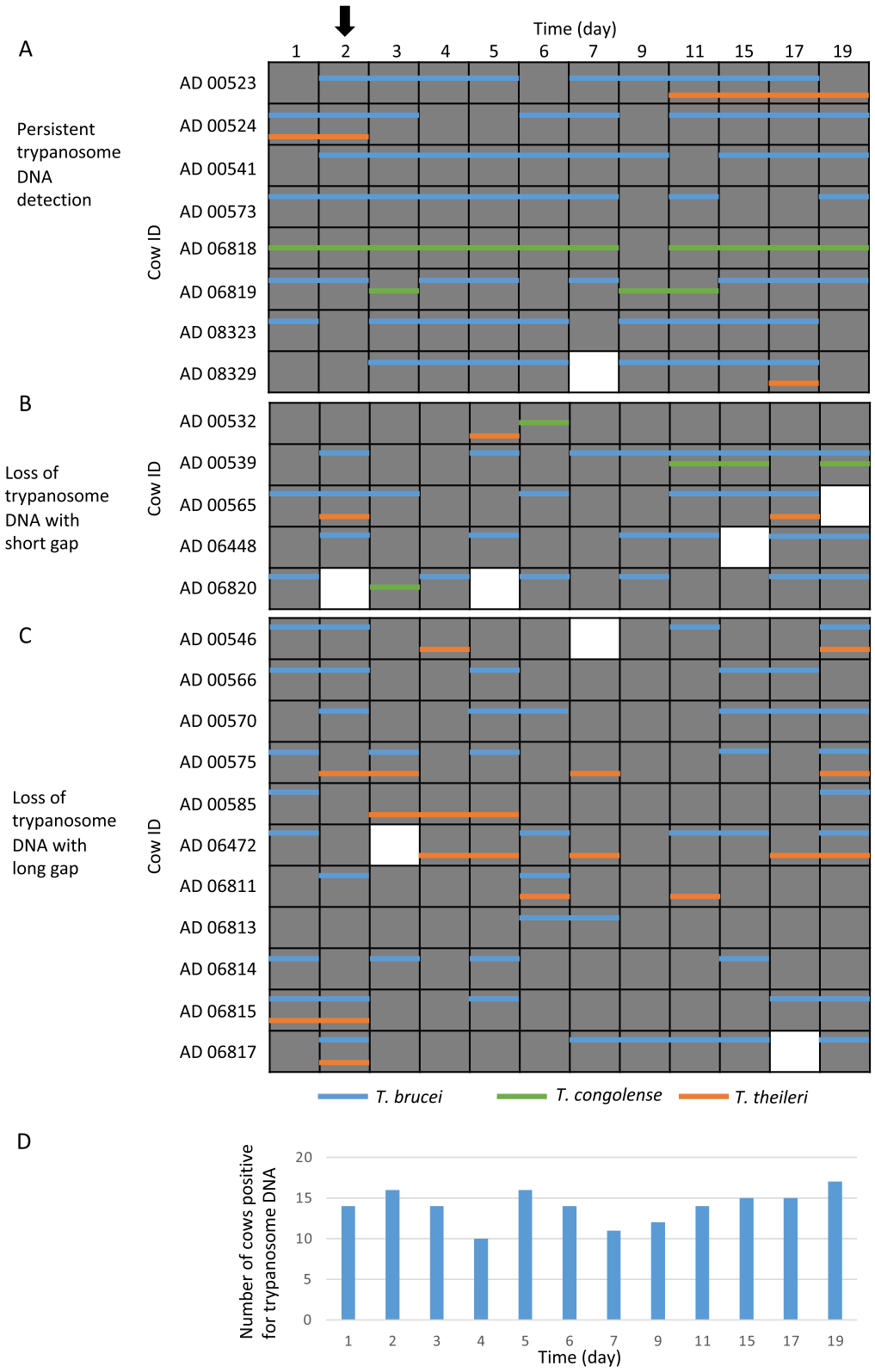

Fig. 1. Pattern of trypanosome DNA detection during diminazene aceturate treatment. Animals are grouped into those that showed (A) Persistence of trypanosome DNA - defined as the same trypanosome species appearing in at least four consecutive time points, (B) Loss of trypanosome DNA with short gaps - defined as the disappearance of same trypanosome species for two sequential time points after treatment with a subsequent reappearance of the parasite DNA and (C) Loss of trypanosome DNA with long gaps - defined as the disappearance of same trypanosome species for three or more sequential time points after treatment with a subsequent reappearance of the parasite DNA. The DNA of each species detected is indicated by a different coloured line. A blank square indicates no sample was taken and a grey square indicates no trypanosome DNA was detected. Black arrow indicates first sample after drug treatment. (D) The frequency of trypanosome DNA detection in all cows at each time point.

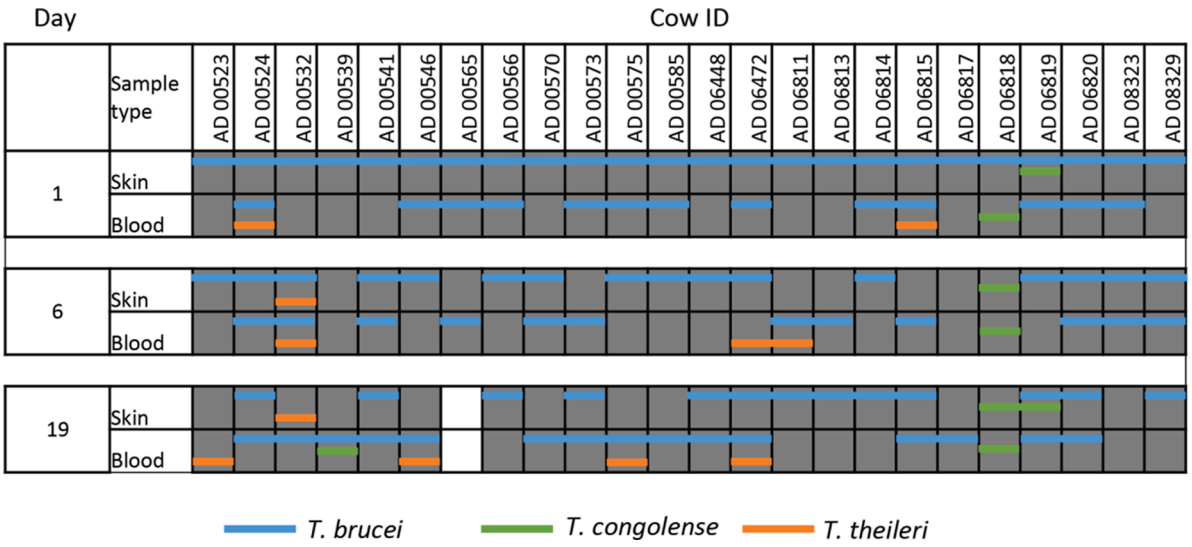

Fig. 2. Comparison of trypanosome DNA detection between the skin and blood. The DNA of each species detected is indicated by a different coloured line. A blank square indicates no sample was taken and a grey square indicates no trypanosome DNA was detected. The selected time points when both skin and blood samples were analysed were days 1 (before treatment), 6 and 19.

infection or treatment response. The cattle ranged from 2-5 years old with greatest number of time points positive for trypanosome DNA (64.0%) seen for the 2 year old cows and the least (51.7%) seen for the 3 year old cows (S3 Table).

4. Discussion

Most studies that investigate trypanosome burden in cattle just provide a snapshot of parasite prevalence and have limited temporal resolution. Here, trypanosome infection dynamics in 24 cows before and after treatment with diminazene aceturate in both the blood and skin were analysed. Unsurprisingly, given this is a farm setting the patterns of trypanosome DNA detection were variable and there was not a definitive clearance seen in any of the cattle. The level of parasitaemia in an infected cow is generally very low and therefore compromises in the diagnostic approach are necessary and the sensitivity of PCR over microscopy was prioritised, as molecular techniques has been shown to be ~30 fold more sensitive than buffy coat (Hounyeme et al., 2022). Furthermore, these low numbers of trypanosome parasites may contribute to the variability that was observed, as the study was reliant on parasites being present in that sample, which will not always occur even in infected cows.

Diminazene aceturate has a long half-life of ~145 hours and therefore over the sampling period the concentration will have only dropped eight fold (Aliu et al., 1993). This means that the concentration will likely have been sufficient to kill susceptible trypanosomes throughout the sampling period. Therefore, the lack of clearance below detectable limits suggest that the trypanosomes species may have developed a level of resistance to diminazene aceturate. These cows were being reared on a commercial cattle farm, which regularly treats its cows with trypanocidal drugs, using mainly diminazene aceturate and less frequently isometamidium chloride; and some of the cows within this cohort have received up to 10 treatments. It is therefore unsurprising that over an extended period trypanosomes resistant to drug treatment would arise. However, despite the evidence of possible drug resistant trypanosomes, there did appear to be a level of clearance as the trypanosomes in the skin samples dropped from being at a level readily detectable to a level at which detection was stochastic. Moreover, the approach in this work relied on a nested PCR which only detects trypanosome DNA and does not indicate whether the trypanosomes are alive or dead. It has been shown that PCR based methods can stochastically detect trypanosome DNA in the blood of patients successfully treated for trypanosomiasis (Ngay Lukusa et al., 2021). Therefore, the continued detection of trypanosome DNA might be due to the slow clearance of dead trypanosomes and their DNA and not the presence of drug resistant parasites. Alternatively, the continued presence of trypanosome DNA could also be as a result of treatment failure due to possible underdosing and/or poor quality/ineffective drug and note that the recommended dose of diaminazene aceturate for treatment of *T. brucei* is double the dose used here.

Interestingly, before treatment all the cows were positive for trypanosome DNA in the skin but ten of these were negative in the blood. Both sampling techniques will only assess the burden in a small percentage of the overall blood volume and skin area; however, given the much smaller proportion of skin sampled than blood makes the 100% positive rate in the skin more striking. The presence of trypanosomes in the skin has recently received renewed appreciation (Trindade et al., 2016), with studies identifying trypanosomes in the skin of patients not positive for trypanosome infection by other methods (Camara et al., 2021). This makes perfect sense as the onward transmission of trypanosomes is through an insect vector that will bite through the skin and therefore future studies of trypanosome prevalence in humans and cattle should give precedence to analysing the parasite burden in the skin over those in the venous blood. Histological analysis of skin samples will be important to confirm the presence of live parasites and providing insight into the importance of tissue tropism during natural African trypanosome infections.

There was limited correlation, when the patterns of trypanosome DNA detection between the skin and the blood were compared, and this is likely a reflection of the fluid interplay between the parasites in the skin and the blood. Indeed, earlier work has shown there was a rapid increase in the level of parasitaemia in the blood of cows infected with *Trypanosoma congolense* and then treated with Berenil (diminazene aceturate) (Mdachi et al., 1995). This highlights that drug treatment will influence the distribution of the parasite in the animal, with those parasites in regions with lower drug concentrations more likely to survive.

Many of the cows in the cohort were infected with two different trypanosome species as has been observed before (Ofori et al., 2022); however, we observed that in most cattle a single species predominates over time and a similar dominance was shown in mouse coinfections with *T. congolense* and *T. brucei* (Silvester et al., 2017). In addition, cows in which there were day-to-day changes in the species of trypanosome DNA detected were observed. These changes towards the end of the observation period may have been due to the infection of that animal

with a new species of trypanosome during the experiment. However, this is unlikely to be the explanation for this pattern at the earlier time points, which suggests that for those animals they were already infected with multiple species before the experiment began. To this picture, an extra layer of variation was added, as the observed a number of cows in which the species of trypanosome DNA detected by PCR in the blood and the skin differed. This observation is likely due to the dynamic nature of the trypanosomes, which are able to move to different regions of the animal but also potentially could reflect a level of tissue tropism.

Male and female cows have a number of different tissues/organs specific to each of them, for example, the testis of the male cows is a potential tissue for the trypanosomes to inhabit. Recent mouse work has shown that *T. brucei* readily infects testes, while *T. equiperdum* a closely related species is sexually transmitted, and disease pathology is observed in the testes (Carvalho et al., 2018; Yasine et al., 2019). The effective drug concentration within the testes is unknown and they may act as a privileged site; however, in this study there was no dramatic differences in the detection of trypanosome DNA between male and female cows.

The Adidome farm at which this study was undertaken has been the focus of two previous studies, one in 2010 (Nakayima et al., 2012) during an outbreak of trypanosomiasis and two more recently (Bakari et al., 2017; Ofori et al., 2022). In the initial study, the dominant species was *T. congolense* and in the more recent studies it was *T. vivax* and now in the current study *T. brucei* was the most common species detected. Over this period, there has been continued chemotherapy of the cows and the use of tsetse control measures and this has likely influenced the most commonly detected trypanosome species, selecting for those able to survive and be transmitted under these conditions. However, the 2010 study used a diagnostic PCR that detected the internal transcribed spacer of the ribsosomal DNA, whereas the latter studies targeted the tubulin gene array and these different approaches may have different detection sensitivities for the different trypanosome species.

5. Conclusion

In summary, the data show that despite treatment of the cattle with diminazene aceturate there was persistent detection of trypanosome DNA, with parasite DNA detected in both the skin and blood across the study. Moreover, the approach in this current study puts emphasis on the diagnostic potential of the skin for African trypanosomiasis. By examining both skin and blood samples, the study highlights the variability of a trypanosome infection in a farm setting. The movement of parasite between different tissues in the host is therefore an important factor to consider when examining approaches to both interrupt onward parasite transmission and clear the host of trypanosomes. Future studies will include validation, by using for example histological analyses to confirm the presence of parasites in the skin during natural infections.

CRediT authorship contribution statement

William Ekloh: Investigation, Methodology, Formal analysis, Writing – review & editing. **Jack D. Sunter:** Formal analysis, Resources, Visualization, Writing – original draft, Writing – review & editing. **Theresa Manful Gwira:** Formal analysis, Resources, Visualization, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106721.

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