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MOLECULAR IDENTIFICATION OF TRYPANOSOMES IN WILD TSETSE FLIES CAPTURED FROM KAINJI LAKE NATIONAL PARK, NIGERIA

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ABSTRACT

In order to detect and identify trypanosomes in the wild, and to better understand tsetse-trypanosome relationships, Tsetse flies were trapped from the Kanji-Lake Nation al Park, North-central, Nigeria, using 20 biconical Tsetse traps obtained from Nigerian Institute for Trypanosomiasis, Kaduna. The traps were deployed, covering a 10km distance on the banks of the Oli River at the park. Flies were harvested daily, sorted and preserved in absolute alcohol. A total of 234 flies were trapped in three days comprising159 females and 75 males. The apparent fly density was determined, (Fly/Trap/Day, FTD). DNA extraction was performed on 72 randomly selected flies. Extracted DNA was then subjected to PCR using ITS1 of the ribosomal DNA. Results identified all flies as Glossina tachnoides sp with an apparent fly density (FTD), of 3.93. PCR amplification indicated that 56 (78%) of the flies were infected with Trypanosoma vivax and Trypanosoma congolense (either as single or mixed infections). This infection rate of 78% is considered high and suggests a high level of flies' vectorial capacity. Tsetse fly being a major transmitter of Animal and human Trypanosomiasis, its high vectorial capacity is a key indicator of the prevalence of the disease. The findings of this study therefore present issues of major health concerns because of the potential risk of Trypanosomiasis infection to tourists, livestock and other animals in the park and its buffer zones. A timely intervention to suppress fly population in the park is suggested.

Keywords: Tsetse fly, Vectorial capacity, PCR, Trypanosoma vivax, Trypanosoma congolense, Glossina tachnoides, Apparent fly density

INTRODUCTION

Tsetse fly (*Diptera*: *Glossinidae*) is a haematophagous insect that feeds on vertebrate blood. The fly is the main vector of African trypanosomiasis by transmitting the flagellate protozoa belonging to the genus *Trypanosoma* (Anne et al., 2018).All African trypanosomes are transmitted to the host through the bite of an infected tsetse fly (Betata et al., 2017). Tsetse flies through the cyclical transmission of trypanosomiasis to both humans and animals greatly affect food production, natural-resource utilization, and the pattern of human settlement in Africa as it is one of the most important factors restricting economic development and a major threat to agriculture, and food security in sub Saharan Africa (Masiga et al., 1992, Clement et al., 2016). Unfortunately, it is still one of the most neglected tropical diseases (NTDs) affecting humans and their livestock with devastating health and economic consequences in Africa (Hotez and Kamath, 2009). Two forms of the human disease, Human African Trypanosomiasis, (HAT) also known as sleeping sickness, exist. The chronic form in West and Central Africa is caused by *Trypanosoma brucei gambiense*, while the acute form in East and Southern Africa is caused by *Trypanosoma brucei rhodesiense*. Animal Trypanosomiasis, also known as Nagana, or Animal African Trypanosomiasis, (AAT), is caused by several species of trypanosomes including *Trypanosoma congolense, T. vivax, T. godfrey, T. simiae* and *T. brucei* (Brun et al., 2010; Betata et al., 2017). AAT is widespread across most of the 38 countries of sub-Saharan Africa that are considered endemic for tsetse flies and the

disease is considered to be a major factor limiting agricultural production especially where livestock farming is a key contributing economic sector (Ouma et al., 2000; Florence and Robert, 2016)Furthermore, the infected animals serve as potential reservoirs for re-infection, if not treated, and this is especially the case with wild animals in protected or restricted areas such as National Parks and reserves (Morlais et al., 1998). Both HAT and AAT remain major public health and veterinary threats, respectively, in most of Africa despite more than a century of research, (Morlais et al., 1998). This may be largely due to the long adult life of tsetse and exclusive haematophagy of both sexes (Franco et al., 2014). Despite all the control measures put in place for the control and eradication of tsetse fly in Nigeria, the vectors have continued to spread to new areas (Onyekwelu et al., 2017). However, the WHO Strategic and Technical Advisory Group for NTDs declared a target to control Trypanosomiasis as a public health problem by 2020 and to achieve zero incidences by 2030 with emphasis on control of the vector -Tsetse (Florence and Robert, 2016).

The distribution of tsetse flies is increasingly becoming confined to protected areas such as game parks and reserves. These parks, due to their suitable vegetative cover and array of available host species, act as tsetse breeding sites. This situation has resulted in increased risk and incidence of infection among tourists, game park staff, game animals and livestock in the buffer zones (Neuberger et al., 2014). For this reason, it is important to determine the infection prevalence in sympatric tsetse species so as to identify which species are important in disease transmission. Such data can then be used to inform decisions on control interventions (Kohagne et al., 2010). In addition, infection prevalence data helps scientists to better understand transmission dynamics and detect spatiotemporal trends, both of which have important implications for disease control (Florence and Robert 2016; Ouma et al., 2000). The classical dissection/microscopy technique may have been the only tool available to determine infection rates in the field but it is labour-intensive, requiring skilled technicians and has a low diagnostic sensitivity (Auty et al., 2012) However, Molecular tools have been developed for the identification and characterization of different species of trypanosomes found in tsetse fly. The polymerase chain reaction tools (PCR) based on specific DNA sequences capable of delivering faster, accurate and more specific diagnosis are now available. Furthermore, and in respect to detection of active trypanosomal infection in tsetse vector, a PCR tool, with primers designed for amplification of satellite DNA of the most important African pathogenic trypanosomes has been reported (Masiga et al., 1992).

Because Tsetse fly is a key factor in animal and human Trypanosomiasis epidemiology, a proper identification of the trypanosomes they transmit is a key indicator of the prevalence of the disease. Moreover, the evolution of resistances to trypanocidal drugs makes chemotherapy difficult to sustain for the control of AAT. For these reasons, vector control remains a very important part of an integrated management of AAT.

This work is therefore to use molecular tools and techniques to detect and identify trypanosome infections, and to determine vectorial capacity in wild trapped flies from the Kanji-lake National park, North-central, Nigeria. The expected results shall serve to confirm that Trypanosomes detection using molecular tools is preferable to the use of microscopy. Secondly, data obtained will be an indicator of the potential risk of infection in the wild animals as well as health risk to humans and livestock in the surroundings, as a first step for typanosomiasis studies and surveillance.

MATERIALS AND METHODS

STUDY SITE

The Kanji-lake national park is located on latitude 9°50’ 19’’N, longitude 4°34’ 24’’E. The park is an amalgamation of the old Borgu game reserve (in Niger and Kwara states) and Zurguma game reserve in Niger state. The area covered is a savannah vegetable zone with a total area of 5,340.82sq km and located in the North- central part of Nigeria between Niger and Kwara states. Kanji lake National Park has an abundance of a diverse wildlife population that serves as ready blood meal source to support tsetse flies populations (Fig.1).

TSETSE FLY TRAPPING AND COLLECTION

Twenty Biconical tsetse traps (from Nigerian Institute for Trypanosomiasis Research, Kaduna) were deployed 100 m apart along the bank of river Oli. All traps were baited with acetone released from an amber-coated two- liter plastic container. For each trap, the geographical coordinates were

recorded using a Global Positioning System (GPS). Trapped tsetse flies were collected at 24 hours interval for 3 days, counted, and sorted according to species and sex. Flies were preserved using absolute ethanol and stored at -20 °C until the extraction of DNA. The apparent fly density was calculated as: $FTD = \frac{\text{number of trapped flies}}{\text{number of traps}} \times \frac{\text{number of days}}{1}$

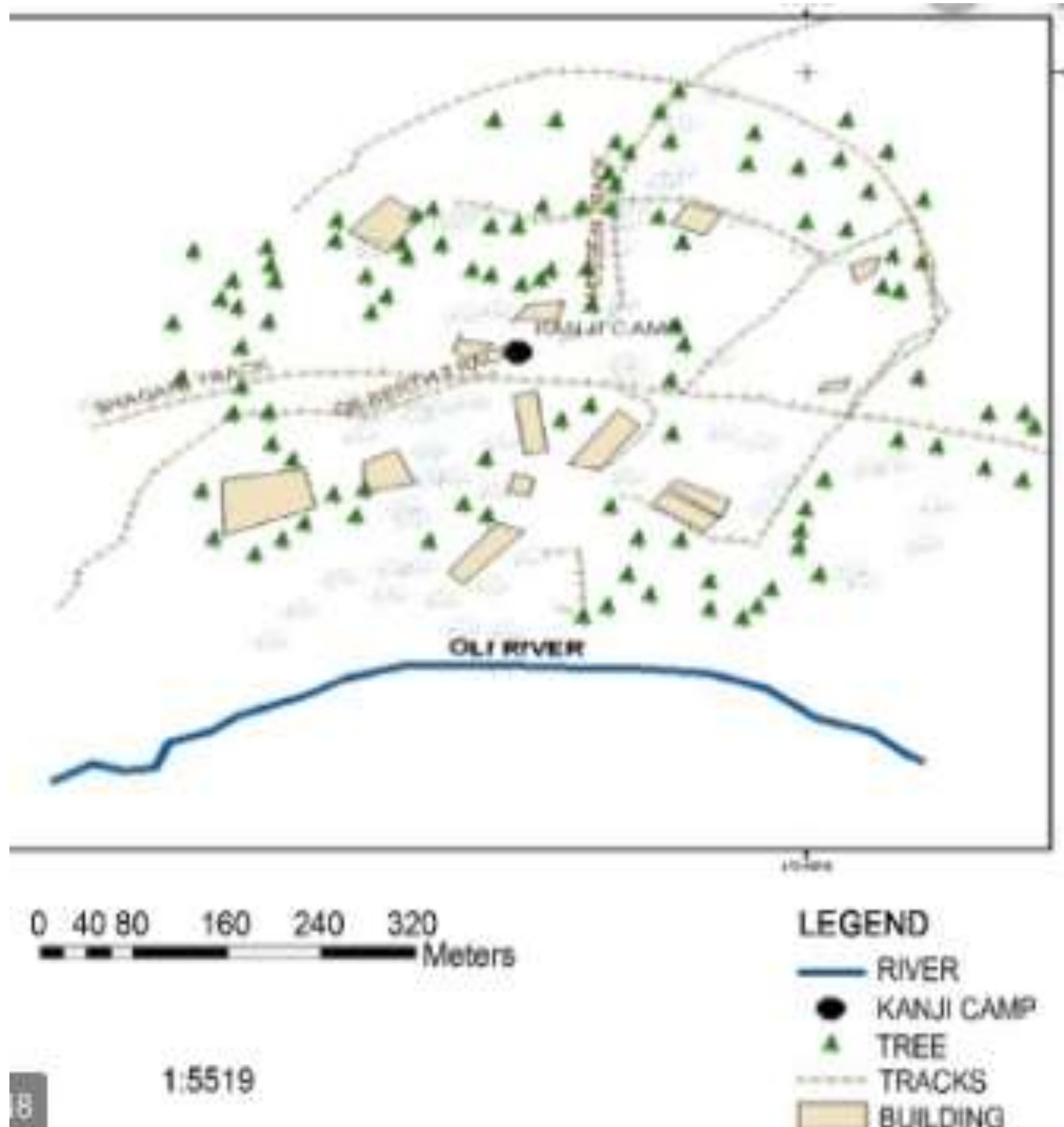

Figure 1: Map showing a Cross section of Kanji lake National park

DNA EXTRACTION

Seventy two randomly selected whole tsetse flies were processed for this study. Flies were washed in distilled water and air-dried. Whole fly was crushed using a pestle and eppendorf tubes, followed by DNA extraction and purification using AccuPrep® Genomic DNA Extraction Kit (Bioneer Inc., South Korea) following the manufacturer's instruction. DNA was precipitated in cold absolute ethanol; air dried and then

dissolved in 20µL of elution buffer and kept frozen at -20°C until PCR analysis was performed.

PCR IDENTIFICATION OF TRPAYNOSOMES DNA USING KIN1&2 PRIMERS

For this identification, two approaches were used: the first approach focused on the internal transcribed spacer 1 (ITS) of ribosomal DNA as described by Desquesnes et al. (2002). Whereas the second approach used primers specific to trypanosome, *T. congolense* forest type, *T.congolense* savannah type, *T. vivax* and *T. simiae* (Desquesnes et al., 2001). For the ITS, the amplification reactions were carried out as described by Konnai et al. (2008). Two pairs of primers designed for ribosomal DNA were used. The first amplification round was performed in a final volume of 25µLcontaining 2µL of DNA extract (template), 20 picomoles of each primer (TRYP18.2C: 5'-GCAAATTGCCCAATGTCG- 3' TRYP4R: 5'-GCTGCGTTCTTCAACGA A-3'), 200 mM of each dNTP and 0.5 unit of Taq DNA polymerase. One denaturing step at 94 °C for 3 minutes was followed by 30 amplification cycles. Each cycle consisted of a denaturation step at 94 °C for 30 seconds, an annealing step at 51 °C for 30 seconds, and an extension step at 72 °C for 2 minutes and a final extension at 72 °C for 10 minutes. The amplified products were diluted 10 fold and 2µL of each dilution was used as a template for the nested PCR. This latter was performed using primers IRFCC (5'CCTGCAGCTGGATCAT 3') and TRYP5RCG (5'ATCGCGACACGTTGTG 3'). The conditions and the amplification program for the nested PCR were identical to those described for the first PCR. Then micro-liters of the amplified products of the nested PCR were resolved on 2% agarose gel containing 10 mg/µL of ethidium bromide. The gels were observed on ultraviolet light and photographed

In this study, Kin1 and Kin2 primers described by McLaughlin et al.(1996) were used. These primers anneal in the conserved regions of the 18S and 5.8S rDNA to amplify the ITS1. Primer sequences were as follows: Kin1, 50-GCG TTCAAA GAT TGG GCA AT-30(reverse); Kin2, 50-CGCCCG AAA GTT CAC C-30(forward).

STATISTICAL ANALYSIS

Results were presented in tables and analyzed as simple percentages.

RESULTS

APPARENT FLY DENSITY

234 *Glossina tachnoides* were trapped comprising159 females and 75 male flies. The average apparent density of 3.93 tsetse flies per trap per day was obtained. (FTD)

PCR IDENTIFICATION OF TRYPANOSOMES

Results of the 72 whole flies analyzed, show that the amplification of internal transcribed spacers of the ribosomal DNA using both the external and internal primers detected 78% (56/72) infected tsetse flies with at least one or two trypanosome species, meaning that trypanosome infections were detected in 78%. The PCR detection using the KIN primers shows that Nannomonas comprising *T. congolense* forest and *T. congolense* savannah had the highest prevalence followed by*, T. vivax, T. godfrey, T. theileri* and *T. b. brucei* were the least in that order. Mixed infections with trypanosome species comprising *T. congolense* savannah and *T. vivax* was recorded accounting for 30% (17/56) of all the positive results. Of the 56 positive samples, the ITS revealed infections by different trypanosome species or subspecies, single or mixed, including 12(21%) single (*T. vivax*), 7(13%) *T. congolense* forest and 8(14%) *T. congolense* savannah type, 17(30%) of mixed infection comprising *T. congolense* savannah type and *T.vivax. O*thers include; 5(9%) *T.godfrey*; 4(7%) *T. simea* and 3(5%) *T.brucei brucei*. See details in tables 1 and 2 below.

Table 1: Showing Primers used in PCR analysis of captured wild fly samples.

Primer set	Primer sequence	Amplicon Size bp	Reference
KIN1&KIN2	5'- GCGTTCAAAGATTGGGCAAT-3' 5'-CGCCCGAAAGTTCACC-3'		McLaughlin et al., (1996)
SPECIFIC Primer			Njiru et al., (2005)
T. congo Forest	F5' GGA CAC GCC AGA AGG TAC TT 3' R5'-GAT TAC GTC CCT GCC ATT TG-3'	500	
T. vivax	F5'-GTG CTC CAT GTG CCA CGT TG-3' R-5'-TCG CTA CCA CAG TCGCAA TCG TCG TCT CAA GG-3'	280	
T. Congo savannah	F-5'-CGA GAA CGG GCA CTT TGC GA-3' R- 5'-GGA CAA ACA AAT CCC GGGCA CA-3'	500	
T. simiae	F-5'- GTG CCC AAA TTT GAA GTG AT-3' R-5'- ACT CAA AAT CGT GCA CCT CG-3'	850	
T. godfrey	F-5'-TGT TTT CTT TTC CTC CGC TG-3' R-5'-GGA AGC AAA AGT CGT AAC AAG G-3'	910	

Table 2: Showing the Results of the detection of Trypanosome species using PCR

	All Trypanosomes	***T.congo* savannah +T. vivax**	***T.congo forest***	***T.congo savannah***	***T. vivax***	***T.godfrey***	***T.simea***	***T.brucei-brucei***
No of Positive/total	56/72	17/56	7/56	8/56	12/56	5/56	4/56	3/56
% of Positive sample	78%	30%	13%	14%	21%	9%	7%	5%

DISCUSSION

In this study, 234 *Glossina tachnoides* were trapped comprising159 females and 75 male flies, with an average apparent density of 3.93 tsetse flies (FTD). Similarly Results of the 72 whole flies analyzed, showed that the amplification of internal transcribed spacers of the ribosomal DNA using both the external and internal primers detected 78% (56/72) infected tsetse flies with at least one or two trypanosome species. The trypanosome infections detected, comprised of *T. congolense* forest and *T. congolense* savannah (with the highest prevalence), followed by *T. vivax,* then *T. godfrey, T. theileri* and *T. b. brucei.* Mixed infections with trypanosome species comprising *T. congolense* savannah and *T. vivax* were recorded also.

Results from this study show evidence of the danger of Animal African trypanosomiasis in the study area, as the identified parasites are animal infective species of trypanosomes (*T. congolense*, *T. vivax* and *T. b brucei*). The infection rate of 78% is a measure of the vectorial capacity of the flies and is considered high. According to Dennis et al.(2014), vectorial capacity is the innate ability of a specific fly species to acquire, mature, and transmit trypanosomes and different tsetse species co- infesting the same habitat can have varying vectorial capacities. This assertion collaborates with the findings of this study in which differential values of infection rates for the different trypanosomes were recorded as follows; (21% *T. vivax*, 13% T. *congolense* forest, 14% *T. congolense* savannah, 9% *T.godfrey*, 7% T. *simea* and 3% *T.brucei brucei*. Trypanosome infections have been detected in several wild animals and wild tsetse flies at varying infection rates at different times in different endemic foci (Djohan et al., 2015) Tsetse flies can acquire mixed infection by feeding on a host that is infected with different species or subspecies (concurrent infection), or by feeding on two or more infected hosts each of which is infected with a different species of trypanosome (sequential infection) (Onyekwelu et al., 2017) The latter may be the case in this study as the Park is populated with a variety of animal species which serve as potential hosts and reservoirs for trypanosomes. Mixed infection could also be as a result of the fact that the development and transmission of different species of trypanosomes may take place in different parts of the tsetse fly depending on species. Since different trypanosome species do not occupy the same parts in the tsetse fly for development and transmission, competition for survival between them will be negligible. These agree with earlier works of Desquesnes and Davila (2002), Dennis et al., (2014) and Onyekwelu et al. (2017).

Secondly, DNA-based methods have shown an advantage of being more sensitive and able to identify trypanosomes to the subspecies level and to detect mixed infections. The identification of mixed infections involving different trypanosome species confirms previous results Desquesnes et al. (2001) and Morlais et al.(1998). The molecular identification of different species of Tsetse fly and trypanosomes (singly and in mixed infection) indicates that the universal ITS targeted PCR method is sensitive, since it has been able to identify six different trypanosomes in this study. With the ITS, different trypanosome species were detected with only two PCR rounds, by generating unique DNA fragment with specific molecular weight. As multiple infections are common in tsetse flies, this technique can be considered very useful in large scale studies aiming to identify natural trypanosome infections as is the case in this study. With this technique, a considerable number of samples can be analyzed in a reasonable time and different trypanosome infections can be revealed, thus achieving a reduction in the cost of analysis. This collaborates with the findings of Onyekwelu et al. (2017) stating that the use of DNA-based methods for identification of trypanosomes is being favored over microscopy.

Detecting and identifying trypanosome species in tsetse flies is surely a good indicator for HAT and AAT, and it is fundamental to understanding the epidemiology of the associated diseases, enabling one to formulate appropriate strategies of efficient tsetse suppression programs, and adapted control methods in the Park.

CONCLUSION

Conclusively, the findings of the present study show that the Kanji-lake national park is a huge reservoir of species and subspecies of African Trypanosomes, the causative agent of African Trypanosomiasis both in terms of parasite abundance and species diversity. This means that game animals, humans and livestock around the park are at risk of being infected and

that a flare up of the disease can occur at any time if control strategies are not immediately put in place.

We therefore recommend that HAT and AAT surveillance as well as vector control programs be put in place in the Park to forestall the incidence of trypanosomiasis

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