



MOLECULAR IDENTIFICATION OF TRYPANOSOMA SPECIES INFECTING DOGS IN NIGERIA BY ANALYSIS OF PARTIAL INTERNAL TRANSCRIBED SPACER-1 OF RRNA GENE

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ABSTRACT

Trypanosomosis is becoming a major health challenge to dogs in the sub-Saharan Africa including Nigeria, but minimal reports are available on the molecular characteristic of trypanosomes infecting dogs in Nigeria. We characterized trypanosomes detected in naturally infected dogs by PCR and sequences analysis of the partial region of the trypanosomes internal transcribed spacer-1 of ribosomal RNA (ITS-1 rRNA). Animals presented to the University of Nigeria Veterinary Teaching Hospital (UNVTH) in Nsukka, Nigeria for examination and treatment were sampled for laboratory tests. DNA was extracted from 21 blood samples obtained from dogs that were confirmed positive for trypanosome infection by microscopy. ITS-1 was PCR amplified and sequenced bi-directionally. Sixteen samples have good bands, though one of them had unreadable sequence. Analyses of the sequence data by BLAST search on NCBI identified *T. congolense*, *T. brucei gambiense*, and *T. evansi* in 4.8, 4.8, and 91.4%, respectively, from the analysed samples from the infected dogs. Although the top BLAST hits for *T. brucei* group were due to *T. evansi* and *T. b. gambiense*, there is not enough discriminatory power in ITS-1 to distinguish subspecies. The aligned sequences of the trypanozoon were less polymorphic. Phylogenetic trees inferred by unweighted pair group method with arithmetic mean (UPGMA) algorithms separated trypanozoon group from the *T. congolense* into two distinct clades. In conclusion, this study suggests that the trypanozoon group of trypanosomes cause more canine trypanosomosis in the study area and suggests inclusion of dogs in strategic planning for control and eradication of trypanosomosis in sub-Saharan African countries.

Keywords: Keywords: Dogs; Internal transcribed spacer; Nigeria; Nsukka; Trypanosomes

INTRODUCTION

Canine African trypanosomosis (CAT) is an important disease of dogs in sub-Saharan African countries (Matete, 2003; Kimeli *et al.*, 2014; Lisulo *et al.*, 2014). In Eastern Nigeria, CAT is responsible for heavy morbidity and mortality of dogs (Omamegbe *et al.*, 1984; Umeakuana *et al.*, 2016). Mortality is usually inevitable if the infected dog is not treated. Previous studies showed that trypanosomosis was responsible for 10.4% of all the canine deaths (Omamegbe *et al.*, 1984) and accounts for the majority of the confirmed haemoparasitic infections in dogs presented for treatment at the University of Nigeria Veterinary Teaching Hospital (UNVTH). *Trypanosoma brucei* and *Trypanosoma congolense* have been reported as the species responsible for CAT in Nigeria (Losos and Ikede, 1972; Abenga *et al.*, 2005). However, it is known that dogs are susceptible to other species of trypanosome including *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, and *Trypanosoma evansi* (Matete, 2003; Gow *et al.*, 2007). A three months study at the University of Nigeria, Veterinary Teaching Hospital (UNVTH) between June and August 1998 in which 55 dogs were screened for the presence of trypanosomes, showed that 15 dogs were positive out of which 14 were due to *T. brucei* and one was a mixed infection of *T. brucei* and *T. congolense* (Umeakuana *et al.*, 2016). *T. brucei* and *T. congolense* have also been identified in *Glossina tachinoides* in Nsukka area of the Eastern Nigeria (Madubunyi, 1987). Local communities living within tsetse belt of Kenya and Uganda have associated potential outbreak of human trypanosomosis (HT) with clinical manifestations and death in dogs (Matete, 2003). *T. evansi* has been reported

in human in India and Vietnam (Joshi *et al.*, 2005; Parashar *et al.*, 2019; Van Vinh Chau *et al.*, 2016). These individuals were confirmed to lack Apolipoprotein-1 (APOL1), a factor that is involved in innate immunity against the *T. brucei brucei* in human (Vanhollebeke *et al.*, 2006). Trypanosomes isolated from naturally infected dogs presented to the UNVTH were usually used for experimental studies within Nigeria (Anene *et al.*, 2006; Omoja *et al.*, 2011; Umeakuana, 2013; Adieme *et al.*, 2014; Eke *et al.*, 2017). Accurate identification of the trypanosome species remains a challenging task in the epidemiology of both human and animal trypanosomosis in tropical Africa (Masiga *et al.*, 1992). Currently, trypanosomes are being identified by DNA-based methods using generic primers that have been designed to amplify the internal transcribed spacer (ITS1) region of the ribosomal RNA gene locus, due to its high copy number and inter-species length variation. Consequently, trypanosome species can be recognized by the unique size of their PCR-amplified ITS-1 region (Desquesnes *et al.*, 2001). This method has been modified with improved sensitivity for detection of trypanosomes in the animal blood (Njiru *et al.*, 2005; Cox *et al.*, 2005; Parashar *et al.*, 2018). These studies have been concentrated on identifying only 58 those trypanosomes that cause disease in cattle, whereas the range of trypanosomes infecting animal is far wider (Adams *et al.*, 2006). In the same vein, the molecular identification of the specific trypanosomes responsible for CAT in and around Nsukka are not well elucidated. Previous studies were based on parasitological tests which are not

sensitive enough compared to molecular techniques. Presently, to the best of my knowledge, only one report of a sequence of diminazene aceturate resistant *T. brucei brucei* isolated from a naturally infected dog presented to the UNVTH has been documented (Anene *et al.*, 2006). There is, therefore, the need to conduct an in-depth study using molecular methods to identify the species and subspecies of trypanosomes responsible for CAT within and around Nsukka, Southeast Nigeria. Hence, this study aimed to identify *Trypanosoma* species in naturally infected dogs at UNVTH using ITS-1 generic primers and sequences analyses.

MATERIALS AND METHODS

Study site and sampled dogs.

Twenty one samples from twenty dogs naturally infected with trypanosomes which were presented to the University of Nigeria Veterinary Teaching Hospital (UNVTH), Enugu State, South-East, Nigeria in Sub Saharan Africa (UNVTH) for veterinary attention between May 2015 and September 2016 were used for this study. The selection criteria included dogs manifesting clinical signs of trypanosomosis, such as pale ocular membrane, corneal opacity, weakness, anorexia, pyrexia, enlarged superficial lymph nodes. Demographic data, signalment (age, sex, and breed), and clinical signs were recorded for each dog, and will be discussed in a different report.

Sample collection and haematology

Blood samples were collected from the cephalic vein of each of the dogs for trypanosome detection using wet film technique and morphological identification of trypanosomes

species on stained thin smear preparations (Murray *et al.*, 1977; Aulakh *et al.* 2005). The blood samples, 5 milliliters each were collected into Ethylenediaminetetraacetic acid (EDTA) coated tubes. Three drops of uncoagulated blood were spotted on FTA® classic card Whatman®, which was air dried and stored in a dry place at room temperature until when needed.

DNA extraction and Molecular identification of Trypanosome

DNA was extracted from the FTA cards using Phenol Chloroform method (GE-Healthcare, 2010). Extracted DNA was stored at -20 °C until needed for PCR. Nested PCR were carried out with a set of outer primers TGC AAT TAT TGG TCG CGC (forward) and CTT TGC TGC GTT CTT (Reverse) followed by inner primers TAG AGG AAG CAA AAG (forward) and AAG CCA AGT CAT CCA TCG (reverse) (Adams *et al.*, 2006). The first PCR was done using the extracted DNA sample as template. The 25- μ L reaction mixture contained 100 ng of DNA, 25 μ M of each primer, 0.3 mM dNTPs, 2.5 μ L 10 \times Dream Taq polymerase buffer, 2 units of Dream Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and water to the final volume. The cycling conditions were 1 cycle of initial denaturation step at 95 °C for 5 minutes; 30 cycles of denaturation at 95 °C for 60 seconds, annealing at 56 °C for 60 seconds, extension step at 72 °C for 30 seconds; followed by final extension at 72 °C and for 4 minutes. The second PCR of the nested was set up using 1 μ L of the first PCR reaction product and the inner primers. All other conditions are the

same except that the annealing time was 40 seconds. The products were electrophoresed for minutes at 100V on 1.5% ethidium bromide-stained agarose gel. Bands were visualized using GelDoc-It2 Imaging System (Analytik Jena AG Jena, Germany).

Sequencing and sequences analysis

PCR products that showed distinct bands were selected and were sequenced bi-directionally using Big Dye Terminator Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). The chromatographs for the sequences were analyzed using MEGA X (Kumar *et al.*, 2018). Sequences obtained were imported into the NCBI database (<https://www.ncbi.nlm.gov/BLAST> and blasted for similarity with other sequences in the Genebank. Multiple sequences alignment of nucleotide was analyzed using Bio Edit software and phylogenetic trees constructed based on unweighted pair group method with arithmetic mean (UPGMA) algorithms using MEGAX software.

RESULTS

Gel electrophoresis, sequencing, and sequence analysis Gel electrophoresis of the 21 Nested PRC products revealed band sizes of 430 bp and 640 bp (gel pictures not included) which corresponded with the expected band sizes of Trypanozoon group and the *T. congolense* sub-group, respectively. Some of the DNA samples were positive to TBR1 and TBR2, Max1 and Max2 but none of the DNA samples were positive to *T. evansi* Type A kDNA mini-circle. Out of the 21 samples from the trypanosome infected dog, sixteen samples had good bands

and were sequenced. Out of the sixteen samples sequenced, one sequence was not readable and as such not included in the analysis. A BLAST search for similarity revealed that one sequence each had a homology of 89% with *T. congolense* (accession number MK132131) and 98% with *T. brucei gambiense* (accession number FN554966) respectively, and thirteen sequences had homologies that range from 97% - 100% with *T. evansi* (accession numbers KX898420 and AB551922). The lengths of the sequences range from 383 bp for *T. congolense*, 363 bp for *T. evansi* and 356 bp for *T. brucei gambiense*. Aligned sequences were shown to be less polymorphic (Figure 1). The phylogenetic trees inferred by UPGMA method revealed tree with topology that separated the trypanozoon group from the *T. congolense* (Figure 125 2). The *Leishmania panamensis* used as an out-group was well separated from the trypanosome's sequences group.

DISCUSSION

Trypanosomosis has been reported in dogs by previous workers (Anene and Omeamegbe, 1984; Omeamegbe *et al.*, 1984; Anene *et al.*, 2006; Umeakuana *et al.*, 2016). It is noteworthy that these earlier studies were based on microscopy and morphological identification of the trypanosomes. Regrettably, these parasitological diagnoses do not provide as a sensitive and accurate identification of the parasites as the molecular based techniques (Njiru *et al.*, 2005; Sharma *et al.*, 2012). Sequence homology search revealed that *T. b. gambiense*, *T. evansi* and *T. congolense* were responsible for canine

trypanosome infection in the sampled dog population. However, the sequence homology 133 with *T. evansi* was not confirmed by species specific test: *T. evansi* Type A kDNA mini-circle could be due to non-representation or non-availability of West African *T. brucei brucei* sequence in the gene bank. This could also be, due to lack of the discriminatory ability of ITS - 1 on the members of trypanozoon group. Similar result has been reported by Umeakuana *et al* (2019) in trypanosome isolates from naturally infected dogs presented for veterinary attention at UNVTH where no samples were identified as *T. evansi* using primers specific for the *T. evansi* Type A kinetoplast DNA minicircle (EVA1 and 2). This was attributed to the highly conserved ribosomal RNA genes in the *Trypanozoon* subspecies which makes it difficult to tell apart the subspecies (Haag *et al.*, 1998; Stevens *et al.*, 2001). A single molecular test able to distinguish between members of *Trypanozoon* subspecies is yet to be developed thus subspecies specific test remains obligatory for their identification (Gaithuma *et al.*, 2019). Gaithuma *et al.*, (2019) reported inability to see any unique cluster that could distinguish 143 between the *Trypanozoon* subspecies which are of high priority because they cause HAT (*T. b. rhodesiense* and *T.b. gambiense*) and secondly, their distribution (*T. evansi* and *T. equiperdum*) is not restricted to Africa. Also, it was not possible to confirm the sub species of *T. brucei gambiense* and *T. congolense* using species specific tests/primers; Tbg1 TgsGp and Tcs satellite repeat, respectively, because of insufficient DNA samples. However, one sequence each has 89% homology with *T. congolense* savannah and *T. brucei gambiense*, respectively. This is in agreement with the recent report by Umeakuana *et al.*, (2019), who reported *T. brucei gambiense*

and *T. congolense* savannah in dogs from this study area using species specific tests. Sequence analysis reveals variation in sequences lengths of the trypanozoon detected. The sequences lengths of 256 bp – 383 bp reported for *Trypanozoon* ITS-1 sequences in this study could not be compared with similar study from dogs due to paucity of data in Nigeria but differ from the 299 bp – 369 bp reported for *Trypanozoon* ITS-1 of ribosomal RNA sequences detected from naturally infected cattle in Nigeria (Takeet *et al.*, 2016). The phylogenetic analysis carried out using UPGMA method revealed a topology which placed the trypanozoon and *T. congolense* in different clades confirming their identities. The clustering together of the trypanozoon species detected in this study is in agreement with the study of Takeet *et al* (2016) where they posited that species entity of *T. evansi* is questionable and as such suggested that *T. evansi* could be a phenotypic variant of *T. brucei*. Detection of *T.b gambiense* in the dog within the study area constitutes a serious public health problem within the area. In conclusion, this study underscores the potential pitfalls of using ITS-1 to identify trypanosomes in tsetse and animals, and suggests that the trypanozoon group of trypanosomes is the predominant species that cause canine trypanosomosis in the study area. The detection of the zoonotic species, *T. b. gambiense* in the dogs from the study area calls for inclusion of the dogs in strategic planning for control and eradication of trypanosomosis in sub-Saharan African countries.

T. congolense.Nsukka10	-----	0
T. evansi-Nsukka-2	-----GGCAACCAAAAAATATACAATATATGTTTTGCATTGTATG	39
T. evansi-Nsukka-16	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	73
T. evansi-Nsukka-9	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	70
T. evansi-Nsukka-7	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	72
T. evansi-Nsukka-13	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	92
T. evansi-Nsukka-11	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	92
T. evansi-Nsukka-14	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	118
T. b. gamb-Nsukka-8	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	104
T. evansi-Nsukka-6	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	98
T. evansi-Nsukka-5	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	98
T. evansi-Nsukka-4	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	98
T. evansi-Nsukka-3	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	98
T. evansi-Nsukka-1	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	105
T. evansi-Nsukka-12	GCATGTATAAATTGCACAGTATGCAACCAAAAAATATACATA-TATGTTTTAC-ATGTATG	98
T. congolense.Nsukka10	-----	0
T. evansi-Nsukka-2	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	99
T. evansi-Nsukka-16	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	133
T. evansi-Nsukka-9	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	130
T. evansi-Nsukka-7	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	132
T. evansi-Nsukka-13	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	152
T. evansi-Nsukka-11	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	152
T. evansi-Nsukka-14	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	178
T. b. gamb -Nsukka-8	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	164
T. evansi-Nsukka-6	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	158
T. evansi-Nsukka-5	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	158
T. evansi-Nsukka-4	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	158
T. evansi-Nsukka-3	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	158
T. evansi-Nsukka-1	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	165
T. evansi-Nsukka-12	TGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATGCTATACATAGTTCTGTTATTTT	158
T. congolense.Nsukka10	-----TAATATATACATATG-----	17
T. evansi-Nsukka-2	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	159
T. evansi-Nsukka-16	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	193
T. evansi-Nsukka-9	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	190
T. evansi-Nsukka-7	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	192
T. evansi-Nsukka-13	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	212
T. evansi-Nsukka-11	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	212
T. evansi-Nsukka-14	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	238
T. b. gamb -Nsukka-8	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	224
T. evansi-Nsukka-6	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	218
T. evansi-Nsukka-5	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	218
T. evansi-Nsukka-4	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	218
T. evansi-Nsukka-3	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	218
T. evansi-Nsukka-1	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	225
T. evansi-Nsukka-12	CTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATACTTTTTAATGCATGCTCTACAT	218

Figure 1: Alignment of Internal Transcribed Spacer 1 (ITS-1) of ribosomal RNA sequences of Trypanosoma species detected in naturally infected dogs in and around Nsukka, Nigeria.

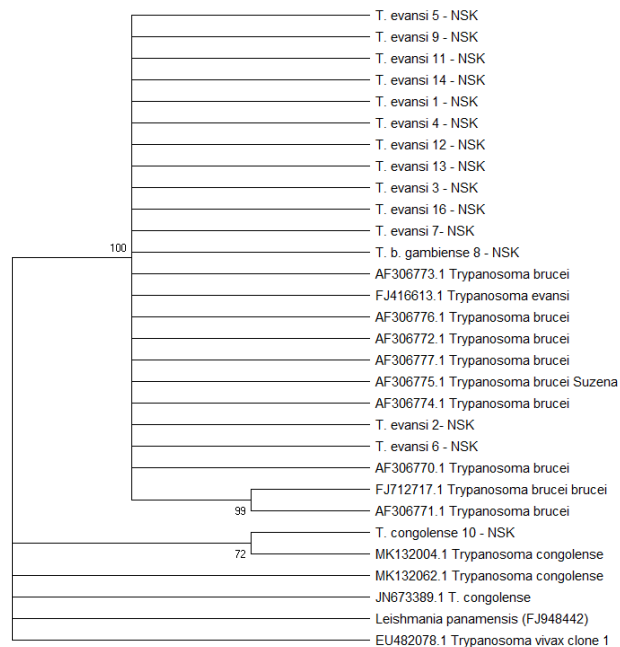


Fig 2: Phylogenetic analysis of Internal Transcribed Spacer 1 (ITS-1) of ribosomal RNA 233 sequences of *Trypanosoma* species detected in naturally infected dogs in and around Nsukka, Nigeria inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

NSK =Nsukka area where the trypanosomes DNAs were isolated

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