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Molecular characterization of drug-sensitive and drug-resistant strains of *Trypanosoma congolense* isolated from Tanzania

Anna F Ngumbi and Ladslaus L Mnyone

Abstract
Like in any other disease-causing agents, intra-species variation in trypanosomes influence how individual subspecies interact with their hosts, vectors and external environment. We conducted genomic characterization and phylogenetic analysis of two stocks of trypanosomes originating from different parts of Tanzania: *Trypanosoma congolense* Mikese, a putatively drug-sensitive strain and *T. congolense* SIO-201 Mbagala, a putatively drug-resistant strain. These strains were isolated and maintained in the laboratory for several years; and have been distinguished from other members of the group and regarded as different strains based on morphometric measurements only. Polymerase Chain Reaction (PCR) using species-specific primers confirmed that indeed the two strains belong to *T. congolense*. Sequencing and phylogenetic analysis revealed further that the two strains are genetically distinct and are related to genotypes described elsewhere in the region. The *T. congolense* drug-resistant strain was identified as *T. congolense* savannah whereas the sensitive strain was identified as *T. congolense* Kilifi. These findings warrant further studies to establish prevalence, distribution and drug sensitivity status of these two genotypes across Tanzania to inform the development of effective control and surveillance strategies.

Keywords: Drug-resistant, drug-sensitive, genotype, *Trypanosoma congolense*

Introduction

The African Animal Trypanosomosis (AAT) remains one of the major causes of poverty and food insecurity in Africa [1]. The AAT causes approximately 3 million cattle deaths annually. The AAT related deaths and other costs related to prevention and treatment correspond to direct annual loss of approximately 1-4 billion US dollars worldwide [2].

The transmission of AAT in Tanzania and elsewhere in Africa is primarily transmitted by tsetseflies [3]. Tsetseflies and the disease are widely distributed in Tanzania. Nevertheless, the epidemiological map of this devastating disease is expanding drastically. Many species of trypanosomes are capable of causing AAT in cattle and other animals; however, *Trypanosoma congolense*, *T. vivax* and *T. brucei* play the most significant role. Of these, *T. congolense* remains the most pathogenic and widespread species [4].

Trypanosoma congolense belongs to the subgenus Nannomonas. Other important species under this subgenus are *T. simiae* and *T. godfreyi* [5]. Their pathogenicity similar to members of other subgenera varies with species of susceptible host animals. *T. congolense* causes the most severe disease in cattle and other susceptible domestic animals other than pigs. *Trypanosoma simiae* and *T. godfreyi* cause acute and sub-acute diseases in pigs [5, 6, 7]. Generally, these three species are quite similar morphologically. However, they can provisionally be separated through their morphometric measurements and isoenzyme electrophoresis. Trypanosoma congolense is the smallest (9.0–22µm in length), followed by *T. godfreyi* (9.1–21.8µm) and *T. simiae* (12–24µm). Furthermore, all three species are morphometrically smaller than other trypanosomes of animals, for instance, *T. vivax* (18-26µm) and (*T. brucei* 17–30µm) [8]. The two closely related to the three species, *T. congolense* and *T. simiae* can be distinguished further through isoenzyme electrophoresis [9]. However, this method is incapable of differentiating subspecies/genotypes within *T. congolense* and *T. simiae*. Molecular techniques are increasingly becoming desirable in addressing such and other prevailing challenges. Polymerase Chain Reaction (PCR) has made explicit identification of subspecies and/or sub-species possible, thus allowing characterization of trypanosomes to the lowest level possible. Intriguingly, genomic sequencing has rendered it possible to identify and/or characterize novel trypanosomes.

Through PCR, two biochemically and genetically distinct clades have been demonstrated within the subgenus Nannomonas: *T. congolense* and *T. simiae* clade [9, 10]. The *T. congolense* clade presently contains three recognized subspecies or genotypes namely *T. congolense* Savannah, *T. congolense* Kilifi and *T. congolense* Forest [6, 11]. The *T. simiae* clade presently contains two recognized genotypes or subspecies namely *T. simiae and T. simiae Tsavo* [12].

Intra-species genetic variations in trypanosomes influence how individual subspecies or genotypes interact with their hosts, vectors and external environment. These and other kinds of variations eventually affect their geographical distribution, pathogenicity, transmissibility and control options. Studies have repeatedly emphasized variations on transmissibility, pathogenicity and virulence among subspecies of trypanosomes [13-19]. We conducted molecular characterization of two stocks of trypanosomes originating from different parts of Tanzania: *T. congolense* Mikese (isolated from cattle in Mikese village, Morogoro region, Tanzania), a putatively drug-sensitive stock and *T. congolense* SIO-201 Mbagala (isolated Mbagala ward, Dar es Salaam region, Tanzania) a putatively drug-resistant strain. These strains were compared with subspecies or genotypes described elsewhere in the region. These strains were isolated and maintained in the laboratory for several years; and have been regarded as subspecies, rather tentatively, without confirmatory molecular characterization.

Materials and Methods

Blood sample collection

Blood samples from infected mice were collected via cardiac puncture using 23 gauge needle and kept in EDTA- coated tubes. Blood samples from infected steers were collected from the jugular vein into 5-ml EDTA-coated vacutainer tubes. The blood samples were labelled accordingly and preserved at 4ºC while awaiting molecular analysis.

Oligonucleotides

The oligonucleotide primer sequences and expected band size used in this study [20] were purchased from Inqaba Biotec East Africa Ltd (Africa's Genomics Company).

DNA extraction

The DNA was extracted from 100µl of each whole blood samples from infected mice and steers using an extraction kit (Quick–gDNA™ Blood MiniPrep kit). This procedure was done following the manufacturer's instructions, except for final elution, whereby 100µl DNA elution buffer was added to elute the DNA. The supernatants containing DNA were either used directly for PCR or stored at -20ºC.

DNA amplification

Identification of trypanosomes was done in three phases. The first phase confirmed the success of trypanosome DNA extraction and allowed for the identification of African trypanosomes. In this phase, the extracted DNAs were used to amplify 750bp fragment of the 18S ribosomal RNA gene of trypanosome using the primers 18ST nF2 and 18ST nR3 (Table 1) specific for African trypanosomes. The second phase was carried out using primers specific to Nannomonas (Table 1). In this phase, a multiplex PCR was done to identify trypanosomes under the subgenus Nannomonas to species level. After the multiplex PCR, DNA samples were further analyzed in the third phase using specific primers designed for detection of two *T. congolense* subgroups ILO 344F/345R and TCK 1F/2R to identify specific trypanosome species to subgroup level. Selected primers were those of a molecular weight corresponding to that of tested DNA fragments. Each PCR round was performed in a final volume of 25µl reaction mixture containing 12.5µl 1X One Taq Master Mix with standard buffer (20mM Tris-HCl [pH 8.9], 22mM KCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 0.2mM of deoxynucleotide triphosphate (dNTPs), 5% glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20,25units/ml of One Taq DNA polymerase), 0.6µl of each primer (forward and reverse) at 10µM, 6.3µl Nuclease free water with 5µl of DNA template. Positive and negative controls were included in each PCR reaction. The reaction mixtures were subjected to a programmable heating block (TaKaRa PCR thermal cycler). The amplification conditions were identical in all phases and involved initial denaturation at 94ºC for 3min, followed by 35 amplification cycles each consisting of denaturation at 94ºC for 1min, annealing at 60ºC for 2min and extension at 72ºC for 30sec, then final extension at 72ºC for 7min.

Table 1: Oligonucleotide primer sequences and their expected product size

Primer specificity	Oligonucleotide sequence	Expected product size (bp)	Reference
African Trypanosomes	18STnF2-5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3'	750	[21]
	18STnR3-5'-TGC GCG ACC AAT AAT TGC AAT AC-3'		
T. congolense Savannah	ILO344F-5,-CGA GCG AGA ACG GGC AC-3'	320	[22]
	ILO345R-5'-GGG ACA AAC AAA TCC CGC-3'		
T. congolense Kilifi	TCK1F-5'-GTG CCC AAA TTT GAA GTG AT-3'	294	[23]
	TCK2R-5'-ACT CAA AAT CGT GCA CCT CG-3'		
T. congolense Riverine-Forest	TCF1F-5'-GGA CAC GCC AGA AGG TAC TT-3'	350	[23]
	TCF2R-5'-GTT CTC GCA CCA AAT CCA AC-3'		
T. congolense Tsavo	ILO892F-5'-CGA GCA TGC AGG ATG GCC G-3'	400	[12]
	ILO893R-5'-GTC CTG CCA CCG AGT ATG C-3'		
T. simiae	TSM1F-5'-CGG TCA AAA ACG CAT T-3'	437	[22]
	TSM2R-5'-AGT CGC CCG GAG TCG AT-3'		

Gel electrophoresis

PCR products obtained were scored as positive for *T. congolense* subgroup after electrophoresis. One percent agarose gel containing 3µl ethidium bromide in 1×TBE buffer was prepared. The electrophoresis chamber was filled with electrophoresis buffer solution until the prepared gel submerged. One microliter of the loading dye was added and mixed with 8 µl of each amplified product. 9µl of each amplification product and DNA ladder of 100bp (9µl) were loaded into the agarose gel wells. The chamber was connected to a power supply of 60 volts and amplification products allowed to migrate for 55-60 min. The amplified DNA products size were detected under ultraviolet (UV) illumination and photographed.

Sequence and phylogenetic analysis

Sequencing was carried out to confirm the correct identification and characterization of the two trypanosome strains. After agarose gel electrophoresis, the PCR products were purified from the gel using a commercial kit (Illustra GFX^{TM} PCR purification kit) as per the manufacturer's protocol. Before sequencing, purified DNA fragments were amplified by cyco-sequencing and followed by ethanol precipitation to remove cyco-sequencing unused components. The amplified DNA fragments were sequenced with the forward and reverse primers using programmed sequencing machine (Capillary Sequencer ABI 3730). Sequences were recorded, aligned, edited and assembled using Geneious software. The sequence results obtained were subjected to BLAST analysis and compared to the sequences available on the NCBI database to identify the highly similar sequences. Phylogenetic analyses were conducted using MEGA7 Software to understand the relationship with other ancestral *Trypanosoma* species and a neighbour-joining tree was constructed.

Results

Identification of the Nannomonas group

DNA for each sample was successfully extracted as revealed by PCR amplification of 18S rRNA. The DNA was further tested for detection of subgenus Nannomonas trypanosomes. The multiplex PCR with Nannomonas species-specific primers showed that both tested samples carried subgroups of *Trypanosoma congolense* under the subgenus Nannomonas (Figure 1)

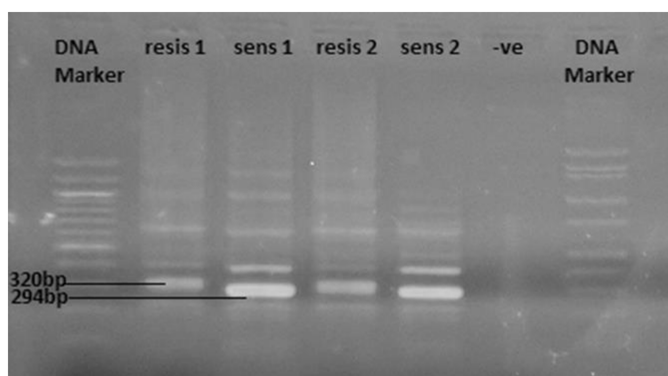

Fig 1: Gel electrophoresis profile of resistant strains lanes 2 and 4 and sensitive strains lanes 3 and 5 and a negative control lane 6; lane 1 and 7 is a 100bp DNA ladder.

Identification of *T. congolense* subgroups

Results were further confirmed by amplification of *T. congolense* to subgroup level using two subgroup-specific primers with a product size that appeared to correspond with the tested samples. Thus two trypanosomes stocks used in this study were identified as *Trypanosoma congolense*. Of these, the *T. congolense* trypanocide resistant stock was shown to contain *T. congolense* savannah genotype while the *T. congolense* trypanocide- sensitive stock contained *T. congolense* Kilifi genotype (Figure 2).

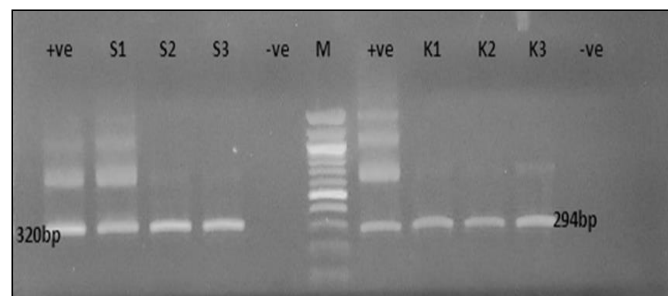

Fig 2: Gel electrophoresis profile showing amplification of positive control for *T. congolense* savannah subgroup in lane 1, resistant strains S1-3 in lanes 2, 3 and 4 and positive control for *T. congolense* Kilifi subgroup in lane 7 and sensitive strains K1-3 in lanes 8, 9 and 10; lanes 6 was a 100bp ladder and lane 5 and 11 were negative controls.

Phylogenetic relatedness of the *T. congolense* strains

Blast analysis of *T. congolense* trypanocide-sensitive and resistant gene sequences confirmed that close matches were found with *T. congolense* Savannah and *T. congolense* Kilifi respectively. Sequences generated in this study were included in the phylogenetic analysis (Figure 3). *Trypanosoma congolense* stocks were grouped into three clusters: I, II and III. Cluster I and II comprised *T. congolense* stocks belonging to the Savannah subgroup while cluster III comprised *T. congolense* belonging to Kilifi sub-group. Phylogenetic results showed that the *T. congolense* trypanocide-resistant stock obtained from cattle at Mbagala Dar es Salaam shared 100% identity with the *T. congolense* IL3000 (savannah type) reference sequences from cluster I and shared 99% identity with *T. congolense* DNA fragment reference sequence from cluster II at the nucleotide level. The *T. congolense* stock obtained from cattle from Mikese shared 99% identity with two *T. congolense* Kenya coast (Kilifi) reference sequences from cluster III.

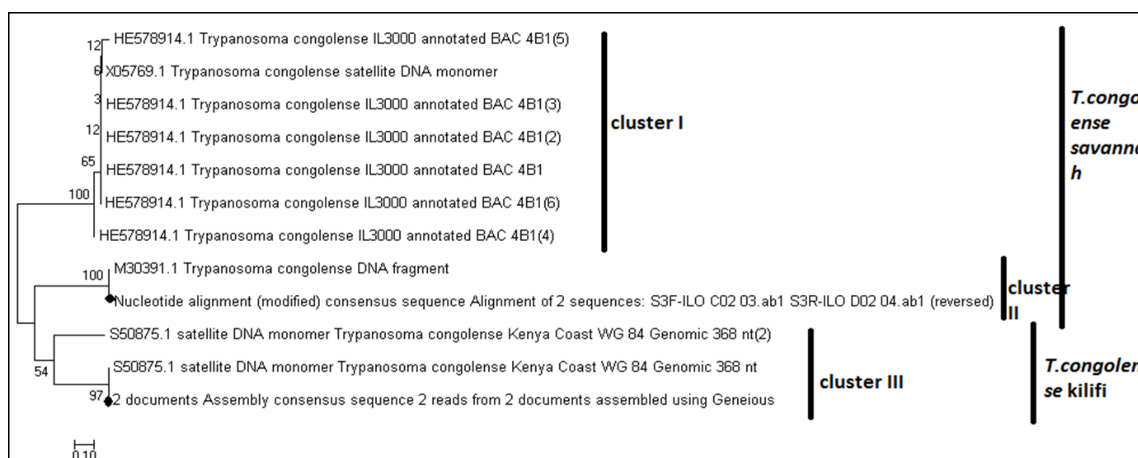

Fig 3: Phylogenetic tree showing relationships of sequences generated from this study shown with black dots and sequences retrieved from database shown with their accession numbers.

Discussion

This study has confirmed that the drug-sensitive and resistant trypanosomes employed in this and other previous studies were all *T. congolense*. These isolates were originally classified as *T. congolense* only based on their small size which is one of the basic characteristics of members of the subgenus Nannomonas [24, 25]. Furthermore, the resistant strain of *T. congolense* Mbagala had a band size of 320bp which was the expected band size of *T. congolense* Savannah type. Phylogenetic analysis of the resulting sequence showed 100% similarity with the Gene Bank reference strain IL3000 which was *T. congolense* Savannah originating from the Transmara region in Kenya [10]. It also showed 99% similarity to *T. congolense* DNA fragment. Similar trypanocidal resistant *T. congolense* was also reported in cattle at Kibaha district, Tanzania [26]. Kibona *et al*. [27] reported drug resistance in north-western Tanzania in mouse infected with human pathogenic trypanosome, *Trypanosoma brucei rhodesiense*. The development and spread of resistant trypanosomes against commonly used trypanocides, isometamidium and diminazene aceturate are quickly developing and/or spreading in Tanzania consequent to indiscriminate use of these drugs [28]. The emergence of single- and multi-drug resistant trypanosome strains is considered a serious concern in AAT control in over 21 African countries, possibly more, including Tanzania [31-32].

Considering that *T. congolense* is the most common species in Tanzania, presence of *T. congolense* Savannah, as suggested in this study, will increasingly spread and impede livestock productivity in the country; more so due to lack of interest by national and international pharmaceutical industries to invest in developing alternative or complementary trypanocidal drugs.

Several studies have associated the occurrence of resistant trypanosomes with indiscriminate use of currently available trypanocidal drugs; such as under dosage and excessive frequency of treatment [2, 32, 33-34]. Arguably, the resistant *T. congolense* Savannah, described in this study, has been associated with the under dosage and frequent exposure of cattle to trypanocidal drugs. These malpractices, under dosage of, and frequent exposure to, trypanocidal drugs have been reported in many parts of Tanzania. The *T. congolense* Savanna was also identified in wildlife species from the Serengeti National park in Tanzania [35] however its response to commonly used trypanocides was not examined.

The *T. congolense* drug-sensitive strain was 97% related to *T. congolense* Kilifi type. The *T. congolense* Kilifi strain was obtained from the coast of Kenya (Kilifi), thus given a synonym Kenya Coast [36]. The Mikese strain was morphologically small, 9-12 micrometer long, with an amplified band size of 294 which was the expected band size about *T. congolense* Kilifi [20]. Despite its wide distribution in East and Southern Africa, less prevalent infections with *T. congolense* Kilifi has been reported in many areas [37]. In Tanzania, *T. congolense* Kilifi has been identified in cattle within human-livestock-wildlife interfaces of Mikumi National Park [38], and tsetse flies from Tarangire and Serengeti National parks respectively [39] as well as tsetse flies from the farming areas of Rufiji district in Coast region [40]. None of these studies indicated a correlation between the presence of the trypanosome in tsetse vectors and infection in cattle.

The molecular characterization has confirmed the existence of two different strains of *T. congolense*, each with its genotype and with variable degrees of sensitivity to trypanocidal drugs. These strains are related to *T. congolense* Kilifi and *T. congolense* Savannah respectively. Equally, the previously observed variation in their morphological length, pathogenicity, transmissibility and therapeutic responses are justified (Ngumbi *et al*. 2017, unpublished data).

Conclusion

This is the first study confirming that indeed the two strains are genetically distinct and relate with subspecies or genotypes described elsewhere in the region. This warrants subsequent studies on their prevalence, distribution and drug sensitivity status in different parts of Tanzania.

Competing interests

The authors declare that they have no competing interests.

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