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Molecular Identification of African Trypanosome Stabilates from Livestock in Lamu County, Kenya

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Abstract
A study was conducted to characterize infectious trypanosomes in domestic animals within Lamu County, Kenya, using parasitological and molecular techniques. Fifteen trypanosome stabilates and 92 whole blood samples collected from parasitologically negative animals were randomly retrieved from the cryobank freezer at the Biotechnology Research Institute, (KALRO-BioRI), Kenya, and characterized. Human serum resistance associated (SRA) gene present in *T. b. rhodesiense* was used to differentiate *T. brucei* positive stabilates. Results showed that 10/15(67%) trypanosome stabilates and 13/92(14%) whole blood samples from cattle, donkeys and goats were positive using PCR. Positive *T. congolense* 5/23(22%) yielded a product size of 700bp using ITS1 primers. The Brucei group 7/23(30%) and *T. vivax* 11/23(48%) amplicons were 480bp and 250bp, respectively. Identified trypanosome stabilates were *T. b. brucei* (7) T. vivax (2) and *T. congolense* Savannah (1). Whole blood PCR profiles revealed 13 isolates namely *T. vivax* (9) and *T. congolense* Savannah *(4)*. *Trypanosoma b. brucei*, *T. vivax* and *T. congolense* Savannah were the etiological agents for AAT in donkeys. In contrast, *T. vivax* and *T. congolense* Savannah caused the disease in cattle, and *T. b. brucei* in goat within Lamu County. The study underscores the significance of molecular and parasitological methods during epidemiological monitoring and surveillance of disease.
Keywords: Trypanosomes, livestock, PCR, Lamu County, Kenya

1. Introduction

African trypanosomes (genus *Trypanosoma*) are protozoan parasites transmitted by an insect, tsetse fly (genus *Glossina*), that is found in 37 countries in sub-Saharan Africa. The parasites cause a group of diseases called African trypanosomiasis (AT), which include sleeping sickness (or human African trypanosomiasis, HAT) in man and nagana (or African animal trypanosomiasis, AAT) in cattle. AAT is caused by *Trypanosoma* (Nannomonas) *congolense, T.* (Duttonella) *vivax* and *Trypanosoma brucei brucei* (Prashant *et al.,* 2005; Sanchez *et al*., 2015). AT is responsible for major socio-economic and public health problems in affected regions. While cases of HAT are decreasing, AAT remains a major challenge, with an annual loss of USD 4.5 billion in rural economies (FAO website) incurred, contributing to food insecurity and consequently poverty and poor standard of living to part of worlds' poorest. Notably, within most of the affected regions, the main economic activity is pastoralism, characterized by continuous movement in search of pasture and water for their livestock.

AAT is caused by *Trypanosoma congolense, T. vivax* and *Trypanosoma brucei*; within each species there are various strains. *Trypanosoma simiae* causes infection in pigs, while in donkeys and camels the disease is referred to as *Surra* caused by *T. evansi* (Prashant *et al.,* 2005). Contrary to other African trypanosome species, *T. evansi* is not known to undergo cyclic development in tsetse, but is mechanically transmitted by biting flies including *Tabanus spp*, *Stomoxys spp.*, *Liperoxia spp* and potentially by tsetse (Gibson, 2003); for this reason the disease has spread outside Africa to Middle East, Latin America, Southern Asia, Australia and Europe (Hoare, 1972). Cases of mixed infections are common, with affected animals showing intermittent fever, anemia, lymphadenopathy and weight loss, loss of body condition (cachexia) and in the absence of treatment sometimes results into death. At livestock production level, the effects of the disease include low fertility rates (caused by poor spermatogenesis, loss of libido, anoestrus and abortion), poor growth rate, stunted growth (Connor, 1994) and low draught power, all contributing to less food and income for livestock keeping communities. Trypanosomosis reduces meat and milk production in cattle by at least 50% (Ashiembi, 2013).

AAT endemic regions generally trypanosome distribution mirror that of vector, and is observed in all these habitats, but HAT, though rare, is only in Western Kenya. As a result of this, studies on the epidemiology

of HAT in humans, animal reservoirs and vector tsetse flies have ideally focussed on these geographic regions (Musa *et al.,* 2005). Notably, Kenyan coast has the largest habitat, infested with *G. austeni, G. pallidipes, G. brevipalpis* and *G. longipennis* (KENTTEC, 2016), and *T. congolense*, *T. vivax* and *T. evansi* infections (Mdachi *et al.,* 2006) reported. The habitat which also has game reserves such as Shimba Hills and forests, is inhabited by communities that have nomadic livestock keeping lifestyles (Bajunis and Somalis) and/or practice mixed farming – both livestock and subsistence crop keeping. Presence of wildlife, domestic animals, vegetation suitable for tsetse breeding and human agricultural activities are among various factors that can influence AAT transmission dynamic and hence are important in disease control and management, and are in of interest.

To understand African trypanosome infection in this habitat, we selected Lamu County, one of the 47 administrative units in Kenya. Most of this region is inhabited by pastoralist community that accesses the forest and more often crosses boarders in search of pasture for their livestock. As well as cows, sheep and goats, the community also keeps donkeys, an animal which there is paucity of data on their infection and consequently potential role in AT transmission dynamic.

2. Materials and methods

a) Trypanosome stabilates

A total of 15 trypanosome stabilates stored at the Kenya Agricultural and Livestock Research Organization-Biotechnology Research Institute- (KALRO-BioRI-) trypanosome bank were used for this study. The stabilates had been collected from infected cattle, donkeys and goats in the Lamu County during epidemiological survey carried out from 2007–2014. More than 26 trypanosome stabilates were prepared directly from blood and subsequently preserved in liquid nitrogen as previously described by (Murilla *et al,* 2014), the bulk from cattle. The 15 trypanosome stabilates used in this study were randomly selected from these stabilates which included 9 from donkeys, 5 from cattle and 1 from a goat. Eight were secondary stabilates (prepared after passaging in mice, restabilated and given the KETRI code) and seven were primary stabilates (not passaged in mice).

b) Whole blood samples

Ninety two whole blood samples in EDTA randomly collected from Donkeys (40), cattle (5) and Goats (47) were used in this study. These samples were part of blood samples collected from parasitologically negative cattle, donkeys and goats that had been brought for observation during the epidemiological study in Kiunga division in 2014. All the whole blood samples were prepared in EDTA and frozen at -20°C.

2.1 Examination of Stabilates for viability and infectivity to white Swiss mice

Two capillaries of each of the 15 stabilates were retrieved from liquid Nitrogen in trypanosome cryobank and placed in a beaker containing ice cubes to thaw slowly. The thawed stabilates were aspirated into 1ml syringe and made up to 0.4mL using EDTA Saline Glucose (ESG) buffer, pH 8.0. After confirming presence of trypanosomes through microscopic examination of wet smears, tentative identification based on morphological characteristics was carried out on the viable stabilates. Each stabilate was then inoculated at 0.2mL of motile trypanosomes in each of two immunosuppressed white Swiss mice intraperitoneally for multiplication. Monitoring for parasitaemia in a drop of tail blood placed on a clean slide was done daily via microscopic examination of a minimum of 20 fields at 400X magnification until parasitaemia reached antilog 8.1-8.4 (Korir *et al*., 2013). Estimation of trypanosome numbers was carried out using the matching method of Herbert and Lumsden, (1976). At peak parasitaemia, blood was drawn from the tail vein using capillary tubes which was then dispensed in a micro centrifuge containing 200µl Tris EDTA buffer (pH 8.0) and this was subjected to PCR analysis. The stabilates that did not grow in mice were directly subjected to PCR analysis.

2.2 Extraction of DNA

The protocol for amplifying nucleated cells (with some modifications) was derived from the Qiagen DNeasy blood and tissue handbook (07/2006). Into a 1.5 ml micro centrifuge tube, 20µl proteinase K was transferred. This was followed by adding 100µl of the whole blood and 200µl Buffer AL to the tube and the mixture was mixed thoroughly by vortexing and then incubated at 56˚C for 10 min. To this sample, 200µl Ethanol (96-100%) was added and mixed by vortexing. The mixture was pipetted into a mini spin column placed in a 2ml collection tube, centrifuged at 8000 rpm for 1 min. The mini spin column was placed in a new 2ml collection tube, 500µl Buffer AW1 added which was then centrifuged at 8000 rpm for 1 min. The mini spin column was then placed in a new 2ml collection tube, 500µl Buffer AW2 added and centrifuged for 3min at 14000 rpm to dry the membrane. The mini spin column was placed in a clean 2ml micro centrifuge tube and 30µl Buffer AE directly pipetted onto the centre of the column. This was incubated at room temperature for 1 min. Discs were then separated from elutes containing DNA through centrifuging at 12000 revolutions/min for 1 min (Mpho, 2008). The DNA was stored either at 4˚C if it was to be analyzed within 3 days or at -20 ˚C if it was going to be analyzed after 3 days.

2.3 PCR amplification of extracted DNA

All samples were first analyzed using ITS1 CF, 5' (CCG GAA GTT CAC CGA TAT TG) and ITS1 BR, 5' (TTG CTG CGT TCT TCA ACG AA) primers (Njiru *et al*., 2005) of the ribosomal DNA which is known to be a suitable target for PCR based detection of all trypanosomes. The positive samples by ITS1 were then analyzed for presence of *T. brucei, T. congolense* and *T. evansi.* The latter was further analyzed to detect presence of *T. evansi* A and B using specific primers EVA A, 5' (ACA TAT CAA CAA CGA CAA AG) and B, 5' (CCC TAG TAT CTC CAA TGA AT) for type A which were earlier designed for the minicircle DNA sequence (Njiru *et al*., 2006). Screening for *T. congolense* involved using species-specific primers TCS1 , 5' (CGA GAA CGG GCA CTT TGC GA), TCS2, 5' (GGA CAA ACA AAT CCC GCA CA): TCK1, 5' (GTG CCC AAA TTT GAA GTG AT), TCK2, 5' (ACT CAA AAT CGT GCA CCT CG) and TCF 1 , 5' (GGA CAC GCC AGA AGG TAC TT), TCF 2, 5' (GTT CTC GCA CCA AAT CCA AC) to further classify it into Savanna, Kilifi and Forest subtypes respectively. *T. brucei* was analyzed using TBR 1, 5' (GAA TAT TAA ACA ATG CGC AG) and TBR2, 5' (CCA TTT ATT AGC TTT GTT GC) primers (Hoare *et al*., 2005). The DNA was amplified using GoTaq® Green Master Mix, (Promega Co. USA) in a 10 µl total volume. Each reaction included 0.2 µl GoTaq® (5 U/ µl), 1 µl PCR buffer (10X), 0.2 µl dNTPs (10 Mm), 0.6µl $MgCl_2$ (25 mM), 1 µl of 10 mM of each primer (10 µM), 4 µl RNase-free water and 2 µl extracted template DNA. Thermocycling for ITS1 PCR profile started with initial hold for 5 min at 94°C, followed by 35 cycles of 94°C for 40 sec, 58°C for 40 sec and 72°C for 90 sec and final extension step of 5 min at 72°C (Njiru *et al*., 2005). These repeated cycles of temperature shifts were done automatically in a thermo cycler, a machine that sequentially shifts between the desired temperatures and remains at each temperature for a specified length of time (Michael *et al.,* 2014).

In thermocycling *T. evansi*, PCR profile started with initial hold for 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and final extension step of 5 min at 72°C. A positive and a negative control for *T. evansi* A was used during the analysis.

Cyclic conditions for *T. congolense* Savannah*, T. congolense* Kilifi and *T. congolense* Forest subtypes using a standard PCR profile started with initial hold for 1 min at 94°C, followed by 30 cycles of 92°C for 30 sec, 60°C for 45 sec and 70°C for 45 sec and final extension step of 4 min at 72°C. In each case, a positive and a negative control were used during the analysis. *T. congolense* Savannah-specific satellite DNA gives a band at approximately 316 bp with species-specific-primersTCS1 and TCS2 which distinguishes it from other *T. congolense* subtypes (Masiga *et al*., 1992, Thumbi *et al.,* 2008, Clement *et al*., 2016).

For *T. vivax*, TV East Africa type universal primers were used with thermocycling starting with pre-denaturation at 94°C (3 min) followed by 35 cycles of denaturation at 94°C (45 s), annealing at 58°C (30 s), extension at 72°C (60 s) and further extension at 72°C (10 min) (Mwandiringana *et al.,* 2012).

PCR products were electrophoresed through 100mls of 2% agarose gel in Tris-acetate EDTA buffer containing 5µl Ethidium bromide dye (10 mg/ml) at 60V for 1hrbefore being visualized under a UV light transilluminator and photographed in an UVTEC gel imager. A negative control and a positive control for each genomic DNA were included in all PCR.

2.4 SRA gene determination - DNA extraction, PCR analysis and gel electrophoresis

Samples that were positive for T.*brucei* group were further analyzed using SRA specific primers SRA A , 5' (GAC AAC AAG TAC CTT GGC GC) and SRA E , 5' (TAC TGT TGT TGT ACC GCC GC).These samples were amplified using GoTaq® Green Master Mix, (Promega Co. USA) in a 10 µl total volume. Each reaction included 0.2 µl GoTaq® (5 U/ µl), 1 µl PCR buffer (10X), 0.2 µl dNTPs (10 Mm), 0.8 µl $MgCl_2$ (25 mM), 1 µl of 10 mM of each primer (10 µM), 3.3 µl RNase-free water and 2.5 µl extracted template DNA. Thermocycling of SRA PCR profile started with initial hold for 3 min at 95°C, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min and final extension step of 2 min at 72°C. A positive control *T. b. rhodesiense* (KETRI 2537) was used during the analysis to compare with the results from samples. The amplicons were identified through gel electrophoresis, visualized under an imager and the results recorded.

3. Results

3.1 Viability and infectivity of Lamu stabilates in Swiss White mice

The identity of these stabilates is shown in Table 1. Based on motility, 7 out 15 stabilates were viable. The species identity of four of the seven viable stabilates was similar to their species identity during isolation in the field. The difference in species observed in the 3 of the seven stabilates is attributed to the initial passage in mice which selected for *T. brucei*. This is an indication that the 3 stabilates may have been prepared from multi infection primary stabilate. Of the 7 viable stabilates only four grew in mice (KETRI 4032, KETRI 4028, KETRI 3984 and KETRI 3985).

Table 1: Stabilates identity, host and Trypanosome species identified by microscopy of stabilates isolated from livestock in Lamu.

Stabilates identity	Year of Isolation	Host	Stabilate species during isolation based on morphology	Examination of preserved samples in 2016 by microscopy
				Stabilate species based on morphology
KETRI 4028*	2014	Donkey	*T. congolense*	*T. congolense*
KETRI 3984*	2007	Donkey	*T. congolense*	*T. congolense*
KETRI 3986	2007	Donkey	*T congolense*	*T. brucei*
KETRI 3985*	2007	Donkey	*T. congolense*	*T. congolense*
KETRI 3987	2007	Donkey	*T. congolense*	*T. congolense*
D3	2007	Donkey	*T. congolense*	*No trypanosome seen*
D4	2009	Donkey	*T. congolense*	*No trypanosome seen*
C28	2014	Bovine	*T. vivax*	*No trypanosome seen*
C18	2007	Bovine	*T. vivax*	*No trypanosome seen*
KETRI 4033	2014	Bovine	*T. congolense*	*T. brucei*
C 98	2014	Bovine	*T. congolense*	*No trypanosome seen*
C99	2014	Bovine	*T. vivax*	*No trypanosome seen*
KETRI 3982	2007	Donkey	*T congolense*	*No trypanosome seen*
D 5	2014	Donkey	*T. congolense*	*No trypanosome seen*
KETRI 4032*	2014	Goat	*T. vivax*	*T. brucei*

Legend: C. cattle; D, Donkey; ID, identity, T: Trypanosoma, *Stabilate that grew in mice

3.2 Results of molecular characterization of 4 stabilates that grew in mice

Results from PCR analysis established that all the four stabilates were trypanosome positive, and whose DNA gave a product size of 480 bp on the ITS1 PCR, similar to that for the positive control sample, *T. b. rhodesiense* KETRI 2537 (Figure 1), indicating that these stabilates belonged to the brucei group of trypanosomes. Further analysis on DNA obtained from these four samples revealed that the samples were negative for *T. evansi* (Figure 2) but were positive for *T. b. brucei* (Figure 3).

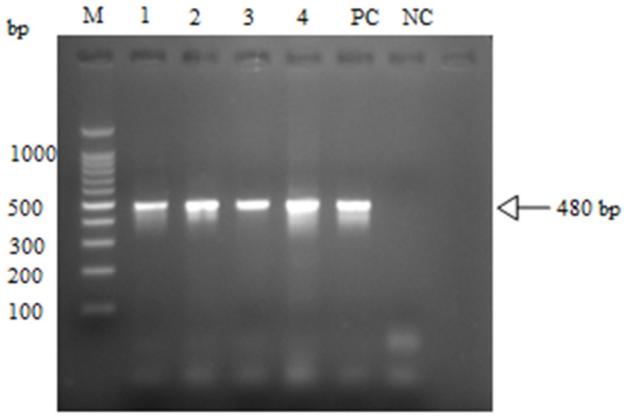

Figure 1: Agar gel electrophoresis of ITS1 PCR products of Lamu trypanosome stabilates that grew in Swiss white mice. M- DNA molecular marker of 100 Bp. Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane 3, KETRI 3984; Lane 4, KETRI 3985 samples; PC, positive control, NC, negative control.

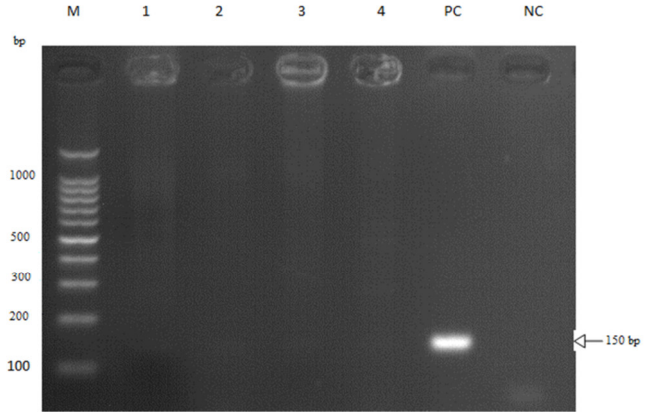

Figure 2: Identification of PCR products using Agar gel electrophoresis. M- DNA molecular marker of 100 Bp. Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane 3, KETRI 3984; Lane 4, KETRI 3985 samples showing negative *T. evansi* products. Included is a positive control (PC) whose image showed one band at 150bp and a negative control (NC).

***T. brucei* analysis**

Samples were further analyzed for presence of *T. brucei* using species specific primers TBR1 and TBR2. All the four samples were positive for *T. brucei* with gel electrophoresis image showing four bands at 177bp (Figure 3). The primers TBR1 and TBR2 target two sites in the satellite DNA and therefore there were two bands but the main one was at 177 bp.

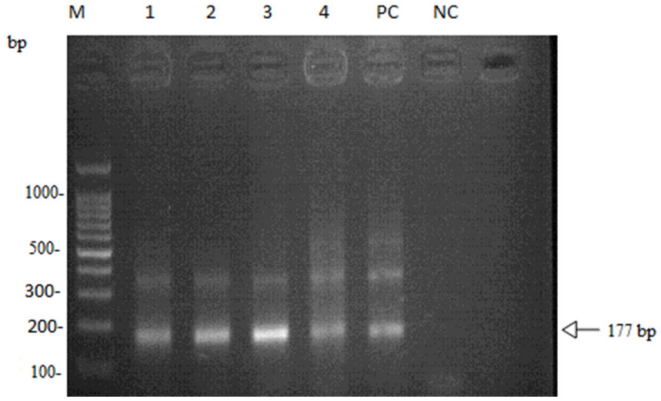

Figure 3: Identification of PCR products of Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane 3, KETRI 3984; Lane 4, KETRI 3985 samples using Gel electrophoresis. Image showed the four samples were positive for *T. brucei*. Also included are M- Molecular marker of 100 bp, PC- positive control and NC- negative control.

3.3 Analysis for 11 samples that did not grow in mice

ITS1 PCR

For the other 11 stabilates which did not grow in mice, six (55%) were positive for trypanosomes when analyzed using ITS1 CF/BR primers (Table 2). Of the positives 3 (50%) had been isolated from donkey and three (50%) had been isolated from cattle. The results for the positive stabilates were as follows; (1/6) 17% were *T. congolense*, (3/6)50 % Brucei group while (2/6) 33% were *T. vivax*. All Brucei group stabilates were from donkeys while *T. congolense* and *T vivax* were from cattle.

Table 2: Trypanosome identity and respective product sizes of stabilates that did not grow in mice.

Lane serial no.	Sample ID	Product size bp	Trypanosome identity
3	KETRI 3982	480	Brucei group
5	KETRI 3986	480	Brucei group
8	KETRI 3987	480	Brucei group
11	KETRI 4033	700	*T. congolense*
6	C 18	250	*T. vivax*
7	C 28	250	*T. vivax*

Legend: C-Cattle, T- Trypanosoma

Figure 4 is an electrophoresis image showing bands at different base pairs of samples that failed to grow in mice. The positive control used was *T. b. rhodesiense* DNA (KETRI 2537).

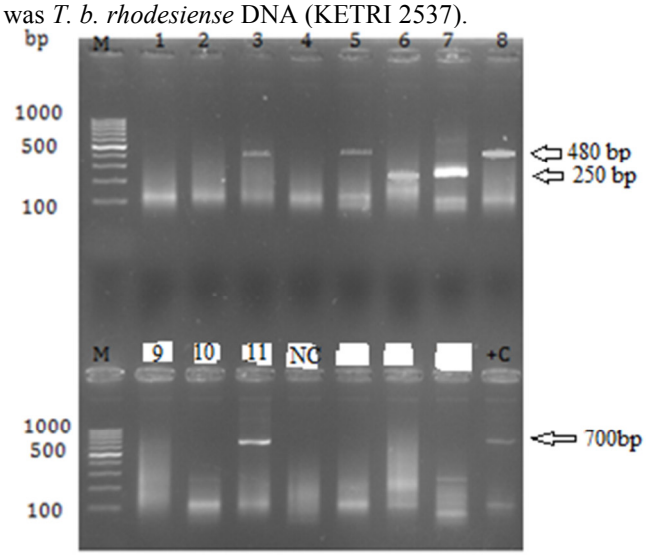

Figure 4: Gel electrophoresis of Trypanosome DNA of samples that did not grow in mice amplified with the ITS1 CF& BR primers**.** Brucei group giving a product of approximately 480 bp, *T. congolense* approximately 700 bp, and *T. vivax* approximately 250 bp. M- Molecular marker of 100bp, +C- positive control and NC- negative control. Lane 3,5 and 8 showed samples KETRI 3982, KETRI 3986, KETRI 3987 isolated from donkeys being positive for Brucei group, lane 6 and 7 showing samples C18 and C28 isolated from cattle being positive for *T. vivax* while KETRI 4033 isolated from cattle was positive for *T. congolense* in lane 11.

Further analysis was carried out on *T. congolense* and Brucei group for more specific classification which showed bands at approximately 316bp and 177 bp confirming presence of *T. congolense* Savannah and *T. b. brucei.* This identified KETRI 3982, KETRI 3986, KETRI 3987 as *T. b. brucei* while KETRI 4033 as *T. congolense* Savannah. The ITS1 PCR results had eliminated the chances of *T. evansi* being present.

3.4 Comparing morphological and molecular characterization of viable stabilates

Comparison of morphological versus molecular characterization of viable (microscopically) stabilates was carried out and results indicated in Table 3. There was agreement in only 2 out of the seven stabilates (28.6%) between the two methods used.

Table 3: Comparison between morphological and molecular characterization of viable stabilates

Isolate identity	Host	Morphological characterization	Molecular characterization
KETRI 4032*	Goat	*T. brucei*	*T. brucei brucei*
KETRI 4033	Cattle	*T. brucei*	*T. congolense* Savannah
KETRI 4028*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3984*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3986	Donkey	*T. brucei*	*T. brucei brucei*
KETRI 3985*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3987	Donkey	*T. congolense*	*T. brucei brucei*

Legend- *stabilates that grew in mice, T- Trypanosoma

3.5 PCR size products for the 92 whole blood samples

a) ITS1 PCR analysis

Ninety two EDTA preserved blood samples were subjected to ITS1-PCR using CF and BR primer pairs, 13/92 samples were trypanosome positive as follows: 9/13 (69%) *T. vivax* and4/13 (31%) *T. congolense* Savannah as shown in Table 4. *Trypanosoma vivax* was only found in donkey blood. Nine out of the eleven positive donkey samples (82%) had *T. vivax* while two (18%) had *T. congolense*. All the trypanosome positive bovine blood (2) had *T. congolense*. No trypanosomes were detected in blood from 47 goats.

Table 4: Trypanosome identity of positive whole blood samples subjected to ITS1-PCR

SAMPLE NO.	ANIMAL OF ORIGIN	TRYPANOSME IDENTITY	SAMPLE NO.	ANIMAL OF ORIGIN	TRYPANOSOME IDENTITY
2	Donkey	*T. congolense*	43	Donkey	*T. vivax*
26	Donkey	*T. vivax*	44	Donkey	*T. vivax*
29	Donkey	*T. vivax*	45	Donkey	*T. vivax*
30	Bovine	*T. congolense*	46	Donkey	*T. vivax*
32	Bovine	*T. congolense*	48	Donkey	*T. vivax*
34	Donkey	*T. congolense*	49	Donkey	*T. vivax*
42	Donkey	*T. vivax*			

The overall infection rate of trypanosome in donkey blood was 27.5% while in cattle blood was 40% and in goats was zero. The infection rate of different trypanosome species identified in different animals whose whole blood samples were as shown in Table 5.

Table 5: Infection rate of different trypanosome species in whole blood samples of livestock in Lamu.

Host	Trypanosome spp.	No. positive	Infection rate (%)
Donkeys	*T. congolense* Savannah	2	5
	T. vivax	9	22.5
	T. brucei	0	0
Cattle	*T. congolense* Savannah	2	40
	T. vivax	0	0
	T. brucei	0	0
Goats	*T. b. brucei*	0	0
	T. vivax	0	0
	T. congolense Savannah	0	0

The electro-micrographs taken after gel electrophoresis are as outlined in figure 5.

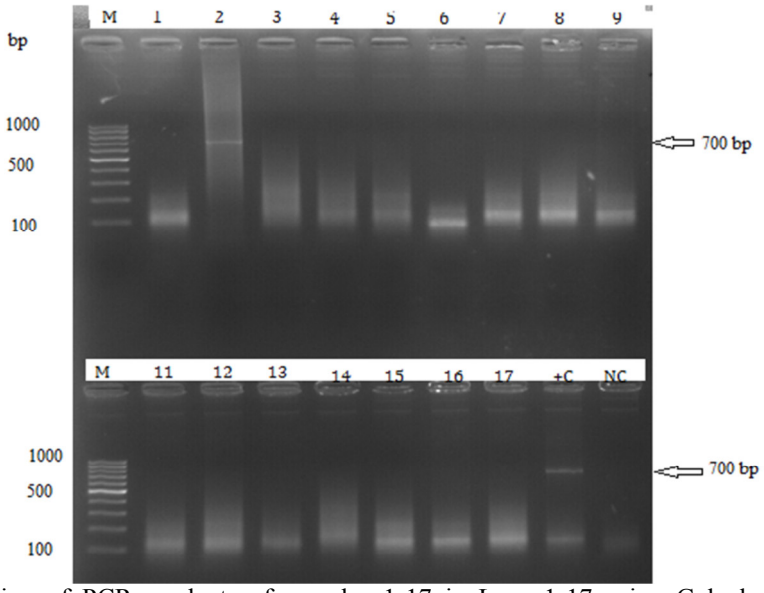

Figure 5: Identification of PCR products of samples 1-17 in Lane 1-17 using Gel electrophoresis. Electro micrograph showed sample number 2 being positive for *T. congolense* at 700 Bp. M- Molecular marker of 100bp, +C- positive control and NC- negative control. All the other samples were negative for trypanosomes.

Samples number 26 and 29 were positive for *T. vivax* at 250 Bp while sample numbers 30, 32 and 34 were positive for *T. congolense* at 700 Bp (figure 6). All the other samples in this gel were negative for trypanosomes.

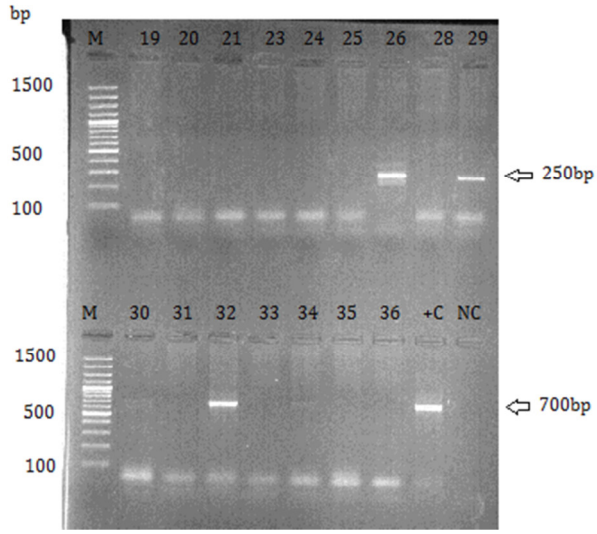

Figure 6: Identification of PCR products of samples 19-36 in Lanes 1-16 using Gel electrophoresis. Image showed samples 26, 29 and 30, 32, 34 positive for *T. vivax* and *T. congolense* respectively. M- Molecular marker of 100 bp, +C- positive control and NC- negative control.

Sample numbers 42-46,48 and 49 were positive for *T. vivax* at 250 Bp. All the other samples in this gel were negative for trypanosomes as electro micrograph (figure 7) shows.

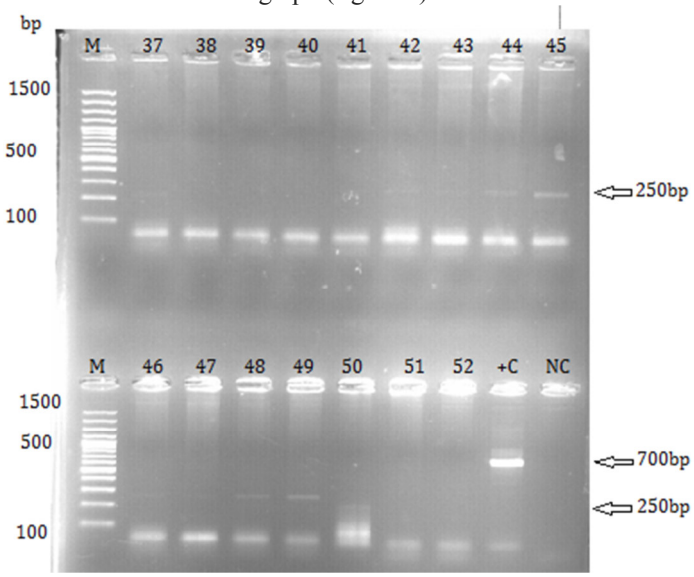

Figure 7: Gel electrophoresis identification of PCR products of samples 37-52 in Lane 1-16 using ITS1. Image showed samples 42-46,48 and 49 being positive for *T. vivax*. M- Molecular marker of 100bp, +C- positive control and NC- negative control.

After ITS1 PCR, further analysis was carried out on *T. congolense* and *T. brucei* sample to classify them using species-specific primers. The image showed bands at approximately 310 bp and 177bp products representing *T. congolense* Savannah and *T. b. brucei* respectively.

***b) T. vivax* analysis**

Further analysis was carried out on the trypanosome positive samples using species-specific primers to confirm the presence of *T. vivax* using universal primers for *T. vivax* East Africa type which gave (figure 8) electro-micro graph with all bands at approximately 700bp. The positive control used (KETRI 2501) gave a product size of 150 bp. Sample 28 was incorporated to confirm its identity since it had an unclear band during ITS 1 analysis. Using species specific primers, it gave a band at 150 bp which failed to confirm presence of *T. vivax* East Africa type.

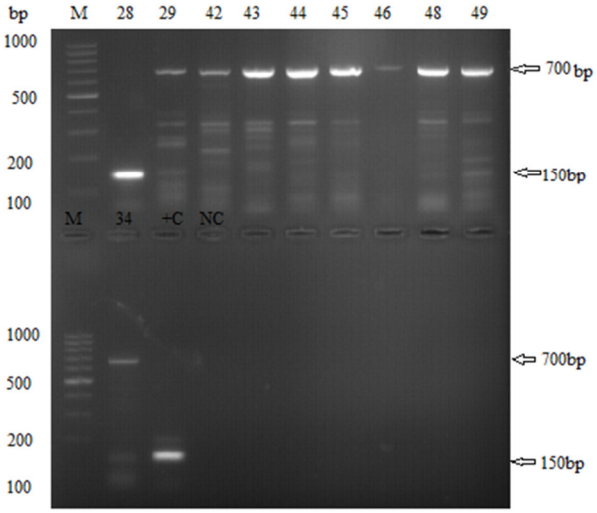

Figure 8: Detection of *T. vivax* positive samples using Universal primers. Representative gel image of electrophoresis of reference DNA samples (+v), test samples 29, 42-46,48,49 and 34 with bands at approximately 700 bp, negative controls (NC) and 100 base pair ladders labelled 'M'. No bands for the negative controls.

c) *T. congolense* Savannah analysis

The samples positive for *T. congolense* in ITS1were further analyzed using species-specific primers TCS1 and TCS2 for Savannah subtype, TCK1 and TCK2 for Kilifi subtype and TCF1 TCF2 for forest subtype. All tested positive for *T. c.* Savannah at 310bp as in the figure 9.

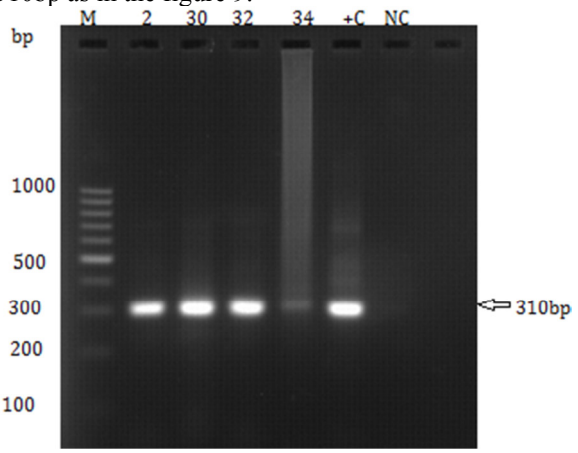

Figure 9: Detection of *T. congolense* Savannah genomic DNA amplified with TCS 1 and 2 primers in trypanosome stabilates. Representative gel image of electrophoresis of reference DNA samples (+C), test samples 2, 30, 32 and 34 being positive for *T. Congolense* Savannah with bands at approximately 310 bp, negative controls (NC) and 100 base pair ladders labelled 'M'. No bands for the negative controls.

3.6 SRA gene determination

Analysis of SRA gene aimed at identifying *T. b. rhodesiense* in all Brucei groups identified. Primers used were SRA A and SRA E and *T. b. rhodesiense* reference DNA (+C) (KETRI 2537 DNA). All samples analyzed were negative for *T. b. rhodesiense* as shown in figure 10. The electrophoresis image showed one band at 460bp representing the reference DNA (+C).

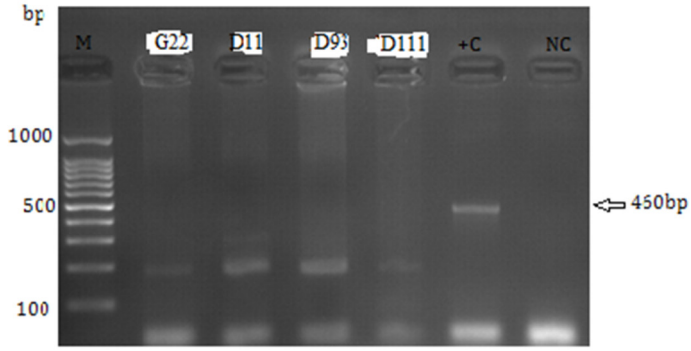

Figure 10: Gel electrophoresis for detection of SRA genomic DNA amplified with SRA A and SRA E primers**.** Representative gel image of electrophoresis showing some Brucei group-positive samples results with a band at 460bp of reference DNA samples (+C), test samples in lane 1-4 labelled KETRI 4032, KETRI 3986, KETRI 3987, KETRI 3982 respectively, negative controls (NC) and 100 bp ladders labelled 'M'.

4. Discussion

Out of the analyzed, 10/15 (67%) trypanosome stabilates and 13/92(14%) negative whole blood samples collected from parasitologically negative animals were positive with trypanosomes by PCR. No mixed infections were observed in whole blood analysis. This is in contrast to earlier work done in Lamu West division of Lamu County where 11 negative blood samples were analyzed by PCR and of the positive samples 67% of them had mixed infections (Mukiria *et al* 2010). This could be attributed to the 5 year difference in the collection of the blood samples between the two studies during which period regular Isometamidium prophylaxis program was in place.

ITS 1 was able to detect *T. congolense*, *T. vivax* and Brucei group in the analyzed samples. A challenge comes when ITS-based tests are carried out singly since ITS has approximately 100-200 copies compared to species-specific tests which usually targets satellite DNA with over 10,000 copies (Njiru *et al.,* 2004) but in this study, species-specific primers were used which identified trypanosomes to subtypes level. Evansi B has only been found in dromedary camels in Kenya and Ethiopia (Njiru *et al.,* 2005) and hence was not characterized in this study. SRA gene present in *T. b. rhodesiense* was not detected in all samples analyzed. We were unable to employ species-specific primers in *T. b. brucei* characterization hence it was identified through elimination method. Identification of *T. vivax* was successful during ITS1 characterization where 11 *T. vivax* were identified with bands at 250bp but using the TV East Africa type universal primers, bands showed at 700bp. Results from this study support other studies suggesting that TV primers target certain DNA sequences that are not conserved in all *T. vivax* stabilates, which resulted to false negatives (McOdimba, 2006, Thumbi *et al*., 2008). In addition, low sensitivity of *T. vivax* observed in this study could be due to the TV primers targeting molecules that are low in copy numbers as compared to ITS-PCR whose target gene could be higher in copy numbers (Jean *et al.,* 1997, Morlais *et al.,* 2001*)*. In a recent study, it was suggested that the species-specific primer sets for *T. vivax* may not accurately assess the level of infection in wild animal's stabilates from Tanzania (Auty *et al.,* 2012). This agrees with another recent study where ITSPCR identified nine *T. vivax* samples that were not identified by species-specific primer set (Ahmed *et al*., 2013).In this study, *T. simiae* which does not usually infect ruminants (Jean *et al.,* 1997) was not considered.

This study highlighted poor resolution in microscopy to detect various trypanosome species in some animals while on the other hand, PCR was not able to pick trypanosome DNA from five samples which were positive by microscopy in the field. Inadequate PCR sensitivity was also observed which could have been caused by factors like loss of DNA during extraction, and low copy numbers of targeted DNA could have led to low sensitivity of PCR. Low parasitaemia also could have led to low sensitivity which is a characteristic of most trypanosome infections as in the case of *T. evansi* and *T. vivax* which develop chronic forms without demonstrable parasites in peripheral blood (Njiru *et al*., 2004). Amongst some samples that showed a negative result though microscopy gave weak PCR amplification which was in tandem with results from Jean *et al.*, 1997 while negative controls of each sample had no bands. Some trypanosome strains were detected by microscopy in the field but not by PCR when they were inoculated in mice which is explained by that they could have been more virulent in livestock but not in mice and otherwise not mouse infective which became difficult to isolate i.e host selection pressure in rodents which eliminated some trypanosome strains studied. Furthermore, since rodents are refractory to *T. vivax* and not all *T. congolense* and *T. brucei* infections become established in a new host (in this case mice), this could have also caused low detection of trypanosomes in mice (Connor and Van den Bossche, 2004).

On the other hand, PCR was able to detect infection in whole blood samples that could not be detected

parasitologically. This increased the overall prevalence of trypanosome infection observed in Kiunga in 2014 survey (data not included) from 10% to 17% and in donkeys significantly from 0 to 24%. Previous PCR analysis of blood parasitologically negative donkey samples showed an infection rate of 81% compared to 3% infection rate found parasitologically in Lamu west Division (Mukiria *et al* 2011). Contamination by heme known to inhibit PCR could have led to the low detection of trypanosomes (24%) in whole blood samples from suspect animals in this study.

Trypanosoma evansi previously isolated from animals in the Kenyan Coast, and which are key in donkeys was not identified in this study. Trypanosome species identified in cattle were also identified in donkeys. The differences in morphological and molecular characterization were evident as established in this study.

5. Conclusion

Out of the species of trypanosomes in Lamu found to infect cattle, goats and donkeys, there were three species identified with a higher prevalence of *T. vivax* observed, followed by *T. b. brucei* and *T. congolense* Savannah. The human infective *T. b. rhodesiense* species was not identified in samples analyzed in this study. Results from this study strongly highlighted importance of using PCR in epidemiological surveys for disease mapping and development of effective control strategies for trypanosomiasis control in livestock.

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