Molecular Detection of Some Bovine Trypanosome Isolates From Different Areas of Sudan

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ملخص البحث

أُستخدمت تقنية تفاعل البوليمريز المتسلسل للكشف على المثقبيات في عينات دم فى أوراق فلايندز الفنية و شركاؤه. أستخدمت 97 عينة دم من أبقار من مناطق مختلفة من السودان، وأجرى تفاعل البوليمريز المتسلسل طبقاً لنظام تنقية لشركة إنفتروجين بيورلينك لمعدات الحامض النووي الرايبوزومى منزوع الأكسجين الجينومى، بإستخدام ثلاث أطقم بادئات. إشتمل الطقم الأول على بادئات محددة للنوع لكل من المثقبية الكونغولية تحت أنواع السافنا و كليفى و المثقبية البروسية و المثقبية النشيطة، وأحتوى الطقم الثانى على بادئات ITS و التى أستخدمت كإختبار مفرد، أما الطقم الثالث فقد أحتوى على بادئات ITS التى أستخدمت فى إختبار تقنية تفاعل البوليمريز المتسلسل التداخلى. تُبين نتائج تفاعل البوليمريز المتسلسل، إضافة الى وجود المثقبية النشيطة و المثقبية البروسية، أن المجموعة المصنفة للمثقبية الكونغولية التى تم التعرف على هويتها تنتمى إلى تحت نوع السافنا. لم يتم الكشف على عدوى للمثقبية الكونغولية تحت نوع كليفى. يؤكد هذا البحث وجود العدوي المختلطة فى الحقل والتى لايمكن كشفها بسهولة بواسطة الطرق الكلاسيكية للكشف على الطفيليات.

Summary

Polymerase chain reaction (PCR) was used for detection of trypanosomes in blood on FTA cards. Ninety-seven blood samples collected from infected cattle from different areas of Sudan were tested. The PCR was carried out according to the protocol of purification of invitrogen Purelink genomic DNA kits, using three sets of primers. The first set included species specific primers to *Trypanosoma (Nannomonas) congolense* subsp Savannah and Kilifi, *T. (Trypanozoon) brucei* and *T. (Dunonella) vivax*. The second set contained ITS primers which were used as a single test and the third set consisted of ITS primers that were used in a nested PCR. The results of the PCR indicate that in addition to the presence of *T. vivax* and *T. brucei*, the only *T. congolense* taxonomic group identified in Sudan belongs to the Savannah. No infection attributable to *T. congolense* Kilifi was detected. This work confirms the existence of mixed infections in the field, which cannot be detected easily by the conventional parasitological methods.

Introduction

Parasitological diagnosis of animal trypanosomosis with low parasitaemia is hampered by low diagnostic sensitivity of traditional detection methods. Accordingly, many serological and molecular tests had been developed to improve the disease diagnosis (Uilenberg, 1998; Eisler *et al*., 2004). Several molecular tools have been developed and widely used for detection and characterization of different trypanosomes species, including DNA probes, species-specific PCR-based assays, restriction fragment length polymorphism (RFLP) analysis, Random Primed Amplified Polymorphic DNA (RAPD), loop mediated isothermal amplification (LAMP), minisatellites and microsatellites analysis, fluorescent fragment length barcoding, and other multiple new techniques (Macleod *et al*., 2001; Desquesnes and Dávila, 2002; Geysen *et al.,* 2003; Simo *et al*., 2005; Masiga *et al*., 2006; Koffi *et al.,* 2007; Adams and Hamilton, 2008). These techniques have provided powerful tools for identification and diagnosis of trypanosomes in their hosts and vectors as well as determining accurate phylogenetic classification. They have also been used to study the epidemiology of bovine trypanosomosis in several countries of Africa (Nakayima *et al*, 2012; Takeet *et al*, 2013; Majekodunmi *et al*, 2013).

Molecular characterization techniques used to detect genetic diversity have a considerable impact on our understanding of the epidemiology of trypanosomosis. It can be used to understand the numerous trypanosome genotypes circulating in different foci. Molecular information can, then be linked with other biological characteristics, such as infectivity, drug resistance and virulence (Gibson, 2001; Agbo, 2003). DNA sequences are increasingly being used for species identification of a wide range of organisms, and the identification of new species; sequences can be directly compared with those from previous studies that are held on publicly accessible databases, such as Genbank at NCBI (Adams and Hamilton, 2008). The accurate identification of the causative organisms of disease is fundamental to the study of epidemiology. Hence, molecular tools are now widely used to detect and to distinguish pathogens. These have greatly improved the understanding of epidemiology for a variety of diseases, including African Trypanosomosis. However, molecular tools for identification and characterization of trypanosome species have not been applied to bovine trypanosomosis in Sudan. Research is now focusing on diagnosis based on the amplification of the internal transcribed spacer-1 (ITS-1) of ribosomal DNA which presents the advantages of being a multi-copy locus (100–200), having a small size (300–800 bp), which varies from one taxon to another but is conserved in size in a given taxon. This may lead to the development of a multi-species-specific diagnostic protocol using a single PCR. By reducing the cost of the PCR diagnosis, this technique would allow a greater number of field samples to be tested in epidemiological studies and/or would increase the variety of *Trypanosoma* species that could be detected. Further investigations are required to develop and optimize multi-species-specific diagnostic tools for trypanosomes, which could also serve as a model for such tools in other pathogens (Desquesnes and D´avila, 2002). Recently a new PCR method, ITS-PCR has enabled differentiation to the species level of some or all trypanosomes circulating in an animal host by means of a single and nested PCR reaction based on identification of internal transcribed regions of the parasite genome (Desquesnes *et al*, 2001, Njuri, *et al*., 2005; Cox *et al*, 2005).

The objectives of this study were aimed at molecular detection of trypanosomes isolated from cattle in different areas of Sudan, and their accurate identification at species levels.

Materials and Methods

Blood samples:

Ninety-seven blood samples on FTA cards were collected from cattle from Blue Nile State (South eastern Sudan), Sennar, Gezira and Khartoum States (central Sudan), South Darfur and North Kordofan States (Western Sudan) and Gedaref State, (Eastern Sudan). Animals were selected for the sampling according to either a previous history of trypanosome infection diagnosed by microscopy or based on a poor observed condition score. Three ml of blood were withdrawn from the jugular vein into a heparinised vacutainer. These samples included samples from previous surveys.

Application of whole blood to Whatman FTA cards

One hundred and twenty microlitres of whole blood were applied directly onto FTA® Cards (Whatman®) which were allowed to thorough dryness prior to storage at room temperature. FTA cards were labelled according to animal No., location and date, and kept in clean polythene envelopes.

DNA extraction from FTA blood samples

Using a 3 mm micro punch, two disc-shaped samples were cut from each of the dried sample spots and placed in a clean RNAse/DNAse free-1.5ml ependorf tube. DNA extraction was performed using commercially available invitrogen purelink genomic DNA kits (Invitrogen Inc.). Briefly, punches were cleaned after every sample using JIK (sodium hypochlorite) and 70% ethanol, flamed using a lighter, and then the punch was used to cut a clean filter paper before cutting the next sample. According to the manufacturer's instructions, 180 µl of purelink genomic digestion buffer, 20 µl of proteinase K stock solution, and 200 µl of lysing buffer were pipetted into 1.5 ml eppendorf tube.The mixture was incubated

at 55 ^{0}C in a water bath with occasional vortexing for 30 minutes. Then, 20 µl RNAse, and 200 µl purelink genomic lysis binding buffer were added to the lysate, mixed well by brief vortexing to obtain a homogenous solution, then 200 µl ethanol was added to the lysate, mixed well by vortexing for 5 minutes to obtain a homogenous solution. The lysate was, then, added to the pure link column with a collection tube and centrifuged at 10000 xg for one minute. The invitrogen column was washed twice using 500 µl of washing buffers W1 and W2, respectively. The column was then placed in a clean 1.5 ml eppendorf tube and the DNA was eluted with 50 µl elution buffer and centrifuged at maximum speed for 1.5 minutes at room temperature; the column was discarded and the purified DNA was kept at -20 °C.

Primers

A list of the primers and the expected size of amplification product obtained with each specific pair of oligonucleotides is given in Table 1 which shows species specific primers and Table 2 which shows ITS primers. These primers were purchased from Bioneer Company (USA).

Table 1: **Trypanosome Species specific PCR Primers**

Species	Primer sequence	Amplification conditions	Product size
Trypanosoma vivax	TVW-1 5'-CTG AGT GCT CCA TGT GCCAC-3' TVW-2 5'-CCA CCA GAA CAC CAA CCTGA-3'	94°C for 3 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 72°C for 5 min (30 cycles)	150bp
***Trypanosoma congolense* Savannah**	TCS 1 5'-CGA GAA CGG GCA CTT TGC GA-3' TCS 2 5'-GGA CAA AGA AAT CCC GCACA -3'	94°C for 3 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 72°C for 5 min (30 cycles)	316 bp
***Trypanosoma congolense* Kilifi**	TCK 1 5'-GTG CCC AAA TTT GAA GTGAT-3' TCK 2 5'-ACT CAA AAT CGT GCA CCT CG-3'	94°C for 7 min, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, 72°C for 5 min (30 cycles)	294 bp
Trypanosoma brucei	TBR1 5'-CGA ATG AAT AAT AAA CAA TGC GCA GT-3' TBR 2 5'-AGA ACC ATT TAT TAG CTT TGT TGC-3'	94°C for 3 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 5 min (30 cycles)	177bp

Table 2: Primers of ITS CF and ITS BR PCR and Nested ITS PCR

PCR primers	Primer sequence	Amplification conditions	Product size
ITS CF and ITS BR PCR (Njiru *et al* , 2005)	ITS1 CF: 5' CCG GAA GTT CACCGA TAT TG 3' ITS1 BR: 5' TTG CTG CGT TCT TCAACG AA 3'	94^{o}C for 5min, (94^{o}C/40sec , 58^{o}C/40sec 72^{o}C/90 sec) (45cycles) 72^{o}C/5min	*T. congolense* Savannah 700 bp *T. congolense* Kilifi 620 bp *T. brucei* 480bp *T. vivax* 250 bp
ITS Nested PCR (Cox *et al*, 2005)	ITS1 5'-GAT TAC GTC CCT GCC ATTTG-3' ITS2 5'-TTG TTC GCT ATC GGT CTT CC-3' ITS3 5'-GGA AGC AAAAGT CGT AAC AAG G-3' ITS4 5'-TGT TTT CTT TTCCTC CGC TG-3'	95 °C for 7min (94 °C for 1min, 55 °C for 1min, and 72 °C for 2min) (35cycles) 72^{o}C/5min	*T. congolense* Savannah 1408-1413bp *T. brucei* 1207–1224 bp *T. vivax* 611- 620 bp

PCR cycling

PCR amplifications were performed in a 25 µl reaction mixture containing 1.5 µl of 25mM $MgCl_2$, 0.5 µl of 10mM mixed dNTPS, 1 µl of 10uM FW primer, 1 µl of 10uM Rev primer, 5 µl of 5x Green GoTaq Flexi Buffer, 0.2ul of Gotaq enzyme (5iu/µl), 2 µl DNA and 15.3 µl of DDH_2O (Promega company, Netherlands). The reaction mixtures were overlaid with paraffin oil to prevent evaporation and subjected to cycles of amplification (Tables 1;2) in a programmable heating

block (Veriti AB Applied Biosystem, programmable thermal controller, USA).

DNA electrophoresis

Two percent gel was prepared for small fragments (0.2–1kb) in one percent TBE (required around 100-200 ml depending on the tray size and number of samples). The first, last or middle well was loaded with a suitable marker according to the expected sample size (a marker of 100 bp Plus Fermentas ladder, Fermantas company, Germany). In addition, the number of samples and a negative and positive control (ILRI, Kenya) were included in the electrophoresis. Eight-ten µl of each sample was loaded in the gel, starting from left to right. Samples were run using a power supply of 100-120 volt for 45-60 minutes. The stock 10x Tris buffer (TBE) was prepared by dissolving 15.76 g of Tris-HCL and 3.72 g of EDTA in 500 ml of distilled water and adjusting the pH to 8.0. The final volume was adjusted to 1,000 ml by distilled water. The 10x TBE was stored at 25 °C. The 1x working solution was freshly prepared by diluting the stock 10x TE buffer with distilled water. All PCRs were performed at Biosciences, Eastern and Central Africa, BECA, ILRI, Nairobi, Kenya.

Statistical analysis

Statistical analysis was performed using SPSS 14. Results obtained using the different sets of primers were compared. The level of significance was considered at $P \leq 0.05$.

Results

Cattle blood collected on 97 cards were subjected to PCR using different sets of primers. All samples were trypanosome positive according to the results obtained using the haematocrit centrifugation diagnostic method (HCT). Most of these samples had very low parasitaemia (only few trypanosomes were seen in the buffy coat).

Trypanosome species were identified firstly using species-specific primers by targeting repetitive DNA for amplification (Table 1). Using these oligonucleotide primers designed to anneal specifically to the satellite DNA monomer of each species/subgroup, we were able to accurately identify *T. vivax* (Fig.1)., *T. brucei* (Fig.2)., and *T. congolense* savannah (Fig.3) in DNA extracted from blood on FTA cards**.** Each species tested produced a strong band of the same size as shown in Table 1, the assay was found sensitive and specific.

No *T. congolense* Kilifi subgroup was found in the samples using species-specific sets of the primers (Fig.4).

Three pairs of primers were used to amplify the ITS-1 region of DNA from FTA blood samples (Table 2). One pair in the single ITS based PCR (Njiru *et al.*, 2005) and Two pairs were used in a nested ITS based PCR (Cox *et al.*, 2005). Results are shown in Figures 5-7 and Tables 3,4 and 5. No significant difference was observed between the three types of PCR.

Table 3: PCR (molecular) detection of trypanosome species in blood of cattle from different areas of Sudan using species-specific Primers

Area	No of samples	*T. v*	*T. c* S	*T. c* K	*T. b*	*T. v* + *T. c* S	*T. b* + *T. c* S	Total Positive
Blue Nile	47	1	35	0	0	1	0	37
South Darfur	15	1	11	0	0	0	1	13
Gedaref	3	3	0	0	0	0	0	3
Gezira	5	4	0	0	0	0	0	4
Sennar	7	3	0	0	0	0	0	3
Khartoum	5	0	0	0	0	0	0	0
North Kordofan	8	0	0	0	0	0	0	0
Unity	7	0	0	0	0	0	0	0
Total	97	12	46	0	0	1	1	60

T. v*, *T. c* S, *T. c* K and *T. b represent *T. vivax*, *T. congolense* Savannah, *T. congolense* kilifi and *T. brucei*, respectively

Table 4: PCR (molecular) detection of trypanosome species in blood of cattle from different areas of Sudan using nested ITS Primers

Area	No of samples	*T. v*	*T. c* S	*T. c* K	*T. b*	*T. v*+ *T. c* S	*T. c* S +T.b	Total Positive
Blue Nile	47	4	30	0	0	3	0	37
South Darfur	15	1	10	0	0	0	1	12
Gedaref	3	2	0	0	0	0	0	2
Gezira	5	3	0	0	0	0	0	3
Sennar	7	2	0	0	0	0	0	2
Khartoum	5	0	0	0	0	0	0	0
North Kordofan	8	0	0	0	0	0	0	0
Unity	7	0	0	0	0	0	0	0
Total	97	12	40	0	0	3	1	56

T. v*, *T. c* S, *T. c* K and *T. b represent *T. vivax*, *T. congolense* Savannah, *T. congolense* Kilifi and *T. brucei,* respectively

Table 5: PCR (molecular) detection of trypanosome species in blood of cattle from different areas of Sudan using ITS CF, BR primers

Area	No of samples	*T. v*	*T. c* S	*T. c* K	*T. b*	*T. v* + *T. c* S	*T. b* + *T. c* S	Total Positive
Blue Nile	47	4	33	0	0	5	0	42
South Darfur	15	1	8	0	0	0	2	11
Gedaref	3	2	0	0	0	0	0	2
Gezira	5	4	0	0	0	0	0	4
Sennar	7	3	0	0	0	0	0	3
Khartoum	5	0	0	0	0	0	0	0
North Kordofan	8	0	0	0	0	0	0	0
Unity	7	0	0	0	0	0	0	0
Total	97	14	41	0	0	5	2	62

T. v*, *T. c* S, *T. c* K and *T. b represent *T. vivax*, *T. congolense* Savannah, *T. congolense* Kilifi and *T. brucei,* respectively.

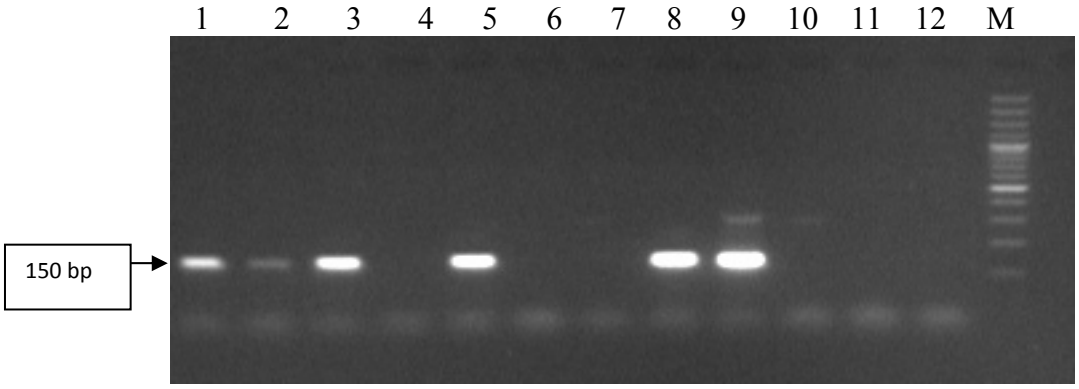

Fig. 1: Detection of *T. vivax* DNA amplified with TVW 1 and 2 primers in field samples. Representative gel image of electrophoresis of DNA samples, test samples labelled 1–12, 100 base pair ladders labelled 'M'. The reference DNA has been amplified with a product size of 150 bp

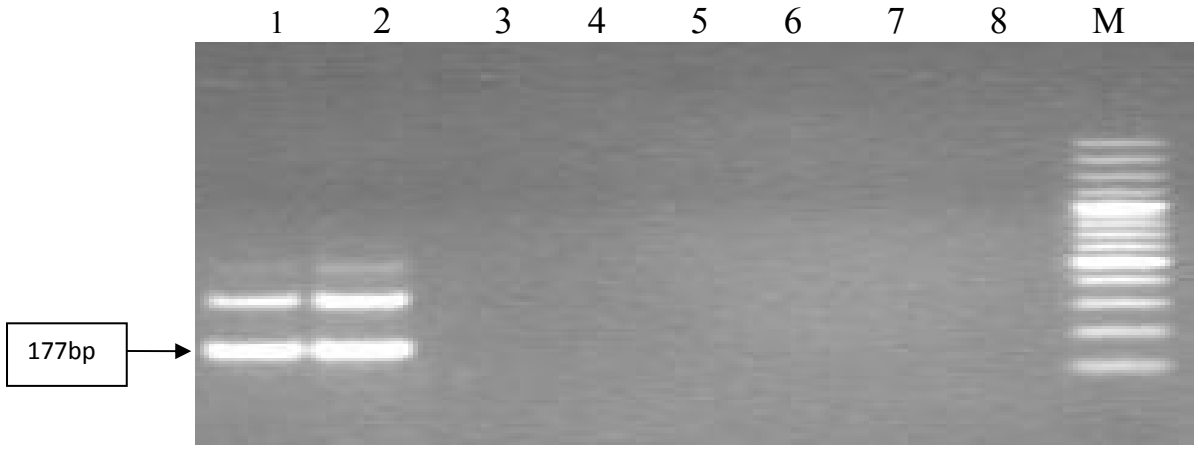

Fig. 2: Detection of *T. brucei* DNA amplified with TBR 1 and 2 primers in field samples. Representative gel image of electrophoresis of DNA samples, test samples labelled 1–8, 100 base pair ladders labelled 'M'. The reference DNA has been amplified with a product size of 177 bp

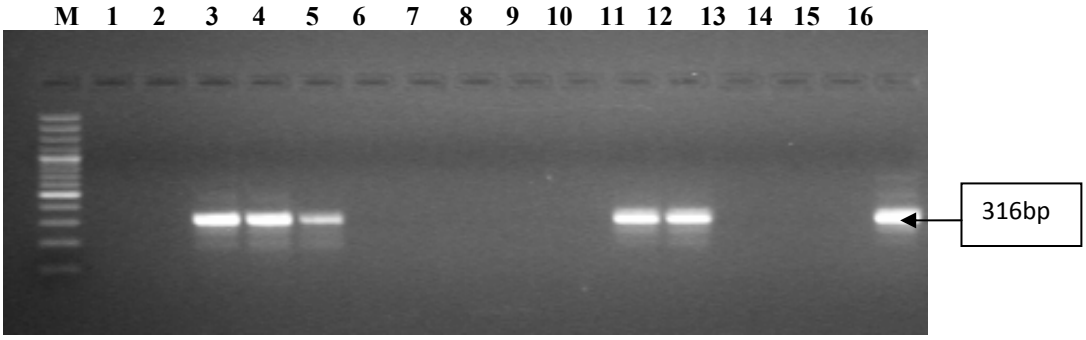

Fig. 3: Detection of *T. congolense* savannah DNA amplified with TCS 1 and TCS 2 primers in field samples. Representative gel image of electrophoresis of DNA samples, test samples labelled 1– 14. 15 is negative control (-v) and 16 is 1180 positive control, 100 base pair ladders labelled 'M'. The reference DNA has been amplified with a product size of 316 bp.

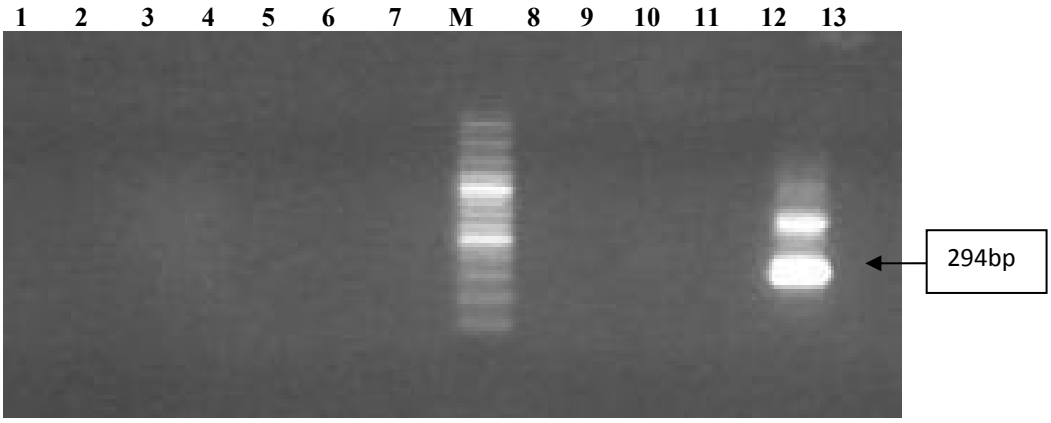

Fig. 4: *T. congolense* Kilifi positive control K45 DNA amplified with TCK 1 and TCK 2 primers. Test samples labelled 1–11; 13-negative control (-v) and 12 is K45 positive control, 100 base pair ladders labelled 'M'. The reference DNA has been amplified with a product size of 294 bp.

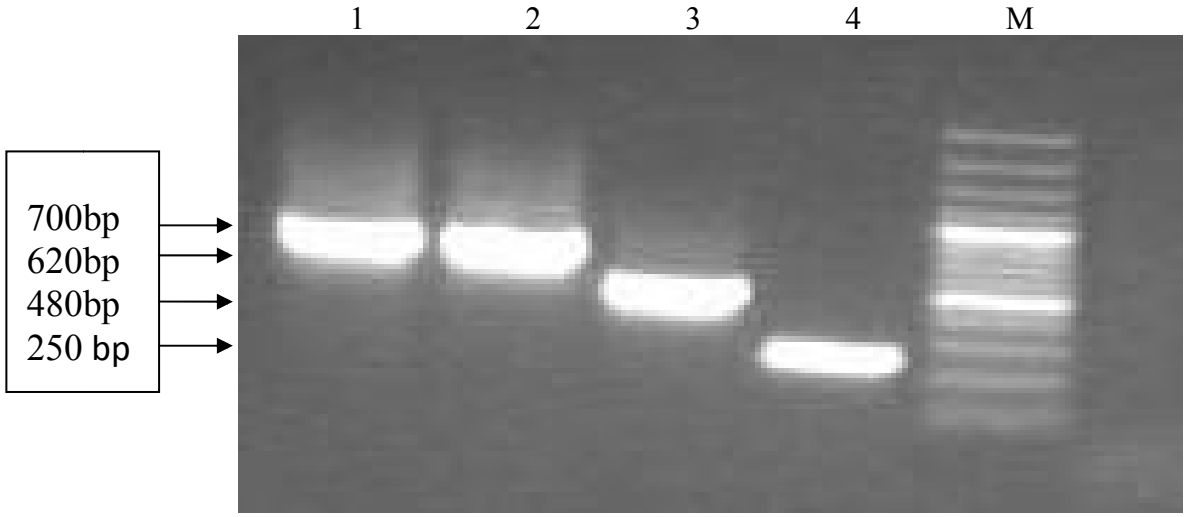

Fig. 5: Gel electrophoresis of Trypanosome control DNA amplified with the ITS1 CF & BR primers. Lane 1= *T. congolense* savannah 1180, lane 2= *T. congolense* Kilifi TC K 45, Lane3= *T. brucei*, lane=4 *T. vivax,* 100 base pair ladders labelled 'M'. *T. congolense* Savannah=700 bp, *T. congolense* Kilifi =620 bp, *T. brucei* =480bp and *T. vivax* =250 bp

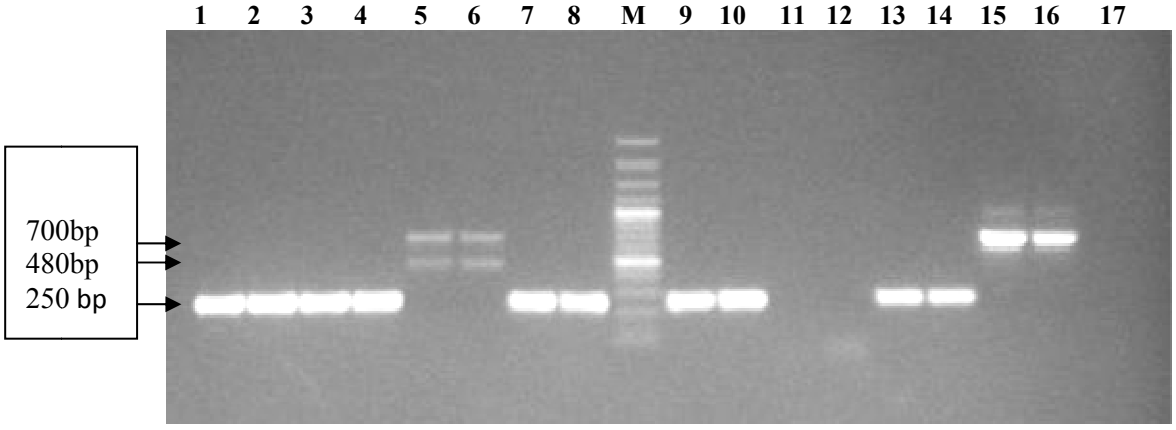

Fig. 6: Representative gel image of electrophoresis of test samples amplified with the ITS1 CF & BR primers. Samples labelled 1–16;1,2,3,4,7,8,9,10,13,14= *T. vivax*; 5,6,15,16 =*T. congolense*; 5, 6= *T. brucei*; 17= negative control (-v); 100 base pair ladders labelled 'M'. *T. congolense* Savannah 700 bp; *T.brucei* 480bp; *T. vivax* 250 bp.

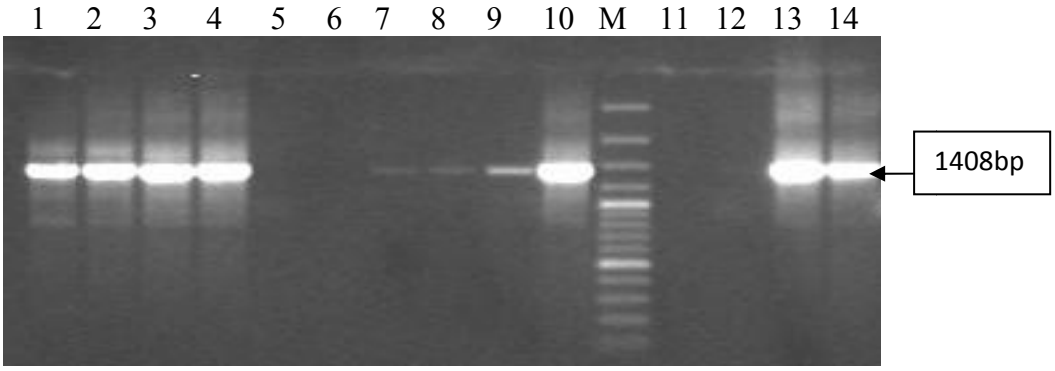

Fig. 7: Representative gel image of electrophoresis of test samples amplified with the nested ITS primers, samples labelled 1–14, 1,2,3,4,7,8,9,10,13,14, *T. congolense* savannah positive samples with a range of 1408 base pair. 100 base pair ladders labelled 'M'.

Discussion

Since the 1980s DNA based technologies including PCR had been used for diagnosis of trypanosomosis (Masiga *et al*., 1992). PCR assays for the detection of trypanosomal DNA in cattle blood dried as blood spots on FTA cards were conducted in this study. Consequently, it is possible to detect evidence of different trypanosome species infections by carrying out PCR on eluates from blood spots.

Although using species-specific primers for trypanosome detection in this study is sensitive and specific, it is costly and time consuming. Therefore, the use of universal tests for all pathogenic trypanosomes is necessary. Desquesnes and Da'vila (2002) reported that the use of universal tests would reduce the cost of PCR three to five times as the number of reactions required per sample would be reduced to one.

The use of ITS CF and BR primers (Njiru *et al*., 2005) enables us to detect more *T. vivax* positive samples (19 positive) compared to the TVW species-specific primers (13 positive). Previously Thumbi *et al*. (2008) found that the test was able to detect more *T. vivax* positive samples. They suggested that the TVW primers target certain DNA sequences that are not conserved in all *T. vivax* isolates resulting into false negatives.

The three tests did not differ in detecting *T. brucei* and *T. congolense* and picked almost equal numbers of *T. brucei*. This is ongoing with the findings of Thumbi *et al* (2008) who reported that the TBR1 and TBR2 primers target a 177bp repeat sequence which occurs in a high copy number of approximately 1000 copies, which explains the comparable sensitivity of these primers with the ITS-based assays.

Although the nested ITS PCR (Cox *et al*., 2005) was effective in detecting trypanosomes, it is laborious, costly and time consuming, besides it was carried out for only two times in this study.

The ITS CF and BR primers were able, in a single PCR, to detect multiple species, from field samples collected in Kenya. However, in this study, only 63 samples out of 97 FTA cards were positive for trypanosomes while the rest of FTA cards didn't work. This may be due to the low parasitaemia of blood collected on FTA cards that lowered the sensitivity of the test due to low quantities of DNA contained. This is in line with the finding of Cox *et al* (2010) who showed that, despite the convenience of Whatman FTA cards and specific PCR-based detection tools, the chronically low parasitaemias in indigenous African zebu cattle make it difficult to establish true prevalence. Although their study specifically applies to FTA cards, a similar effect would be experienced with other approaches using blood samples containing low parasite densities.

The single ITS-based diagnostic assays (Njiru *et al*., 2005) used here detected higher number of positive cases (69) than the four PCR reactions carried out using the species-specific primers, including mixed infections, and reduced the number of PCR reactions per sample to one. These significantly reduced labour, time and cost of carrying out many PCR tests, indicate the superiority of the ITS multi-species detection techniques over the use of species-specific primers. Thumbi *et al*. (2008) advised that reliable epidemiological studies are a prerequisite to designing effective tsetse and trypanosomosis control programmes.

Of the four known subspecies of *T. congolense* (Savannah, Forest, Kilifi and Tsavo), no *T congolense* Kilifi subgroup was found in the samples using all sets of the primers. The two ITS PCR used didn't also reveal any of the forest and Tsavo subgroups and besides, species-specific PCR was not conducted using primers specific for these subgroups in our samples. However, our findings revealed that there is at least only one genotypic group of *T congolense* which is the savannah type; it seems to be the dominant type in south eastern and south western Sudan.

This work confirms the existence of mixed infections in the field, which could not have been detected by the classical parasitological methods.

Although PCR could not differentiate all the 97 FTA card samples as some samples were negative by all PCRs, the authors have found that a small amount of cattle blood on FTA cards filter papers is a useful DNA source for PCR detection of *Trypanosoma* species. Moreover, the FTA cards are suitable for blood sampling from remote areas where power supply or mobile clinics are not available. Gonzales *et al* (2006) reported that in remote places there is no power to use a centrifuge, and taking power generator uses space and it is impossible to take them to farms where cars cannot arrive. Therefore, blood samples prepared on FTA cards make a recommended sampling method for surveillance in remote places, and the lower sensitivity of PCR with this kind of samples compared to PCR with whole blood samples can be adjusted when the true prevalence is determined; this estimation will be considered the test sensitivity. The sensitivity of the PCR can be increased by applying blood directly on the field. It can also be increased by applying buffy coats on FTA cards as well as repeating DNA extraction from the same FTA cards several times using cheap DNA extraction methods to reduce the costs.

The present study ascertains the suitability of using ITS-based PCR assays for large-scale epidemiological studies in Sudan. The three PCR primer sets allow diagnosis of trypanosome infections with reasonable sensitivity and high specificity. However, improving samples preparation and preservation strategy will improve the sensitivity of PCR. The fact that some parasitologically positive samples were found negative with all primers sets, might be due to degradation or improper storage of sample material during transportation to the laboratory. Although the longevity of dead parasite DNA in the host's blood stream is not known, most work suggests that it does not survive for long in the blood (Mamabolo, 2008). So preparing FTA cards just after blood samples collection will help in avoiding the low sensitivity of PCR that result from this fact. Moreover, the very low parasitaemia in the field is a great obstacle to PCR sensitivity. Cox *et al* (2010) have reported that using multiple PCR samples taken from single FTA card are rewarding since a single punch from an FTA card is not sufficient to confirm the infectivity status of an individual animal as parasite DNA is unevenly distributed across the card. They added that at low parasite densities in the host, this stochastic sampling effect results in underestimation of prevalence based on single punch PCR testing. Moreover, preparing FTA cards from buffy coats is by far better in improving the sensitivity of the PCR tests.

Using PCR in this study, the different species of trypanosomes present in different areas of Sudan were determined. *T. congolense* was by far the most abundant species of trypanosomes in the samples, only Savannah-type *T. congolense* was detected. According to Masumu (2006) this genotype has adapted to the various environments in Africa and it is one of the subspecies of *T. congolense* that is commonly found in livestock. *T. congolense* was confined to tsetse infested areas and is not present in the tsetse free areas; *T. vivax* was detected in both areas, which indicates that *T. vivax* can be mechanically transmitted. The findings, also revealed the presence of some mixed trypanosome infections in the field which is difficult to detect using parasitological techniques.

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