Characterisation of the *Trypanosoma brucei rhodesiense* isolates from Tanzania using serum resistance associated gene as molecular marker

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Abstract: Serum resistance associated (SRA) gene has been found to confer resistance to the innate trypanolytic factor (TLF) found in normal human serum; thus allowing *Trypanosoma brucei brucei* to survive exposure to normal human serum. This study was carried out to examine the presence of SRA gene and identify the origin of *T. b. rhodesiense* isolates from three districts in Tanzania, namely Kibondo, Kasulu and Urambo. Twenty-six *T. b. rhodesiense* isolates and two references *T. b. rhodesiense* isolates from Kenya were examined for SRA gene using simple Polymerase Chain Reaction technique. The gene was found to be present in all 26 *T. b. rhodesiense* isolates including the two references isolates from Kenya. The SRA gene was confirmed to be specific to *T. b. rhodesiense* since it could not be amplified from all other *Trypanozoon* including *T. b. gambiense;* and gave an amplified fragment of the expected size (3.9kb), confirming that all these isolates were *T. b. rhodesiense* of the northern variant. Although the geographic distributions of *T. b. gambiense* and *T. b. rhodesiense* are clearly localized to west/central Africa and eastern Africa, respectively, natural movement of people and recent influx of large number of refugees into Tanzania from the Democratic Republic of Congo, could have brought *T. b. gambiense* in western Tanzania. The overlap in distribution of both of these pathogenic sub-species could result in erroneous diagnoses since both trypanosome sub-species are morphologically identical, and currently serologic methods have low specificity. Both the susceptible and resistant *T.b. rhodesiense* isolates possessed the SRA gene suggesting that there is no correlation between drug resistance and presence of SRA gene. The use of SRA gene helps to confirm the identity and diversity of some of the isolates resistant to various drugs.

Keywords: *Trypanosoma brucei rhodesiense*, resistance, SRA gene, PCR, Tanzania

Introduction

Innate protective molecules in the blood of primates influence the host range of African trypanosomes. Human blood, unlike the blood of other mammals, has efficient trypanolytic activity, and this needs to be counteracted by these parasites. *Trypanosoma brucei* consists of three sub-species, non-human infective *Trypanosoma brucei brucei* and human infective *T. b. rhodesiense* and *T. b. gambiense* that are indistinguishable by conventional morphological, biochemical and antigenic criteria but differ by their geographical distribution, host specificity (Mehlitz *et al.,* 1982; Noireau *et al.,* 1989; Hide *et al.,* 1994; Gibson, 2001). A sub-fraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I, apolipoprotein L-I, and haptoglobin-related protein is toxic to *T. b. brucei* but not *T. b. rhodesiense* (Pays *et al.,* 2006).

This question of sub-speciation has been resolved in the past, by inoculating human volunteers with trypanosomes isolated from other animals (Heisch *et al.,* 1958; Onyango *et al.,* 1966), or more acceptably by *in vitro* tests involving incubation of trypanosomes with human blood in the blood incubation test (BIIT) (Rickman & Robson, 1970) or with serum in human serum resistance test (HSRT) (Brun & Jenni, 1987). Biochemical and molecular characterization such as isoenzyme electrophoresis, restriction fragment length polymorphisms using ribosomal DNAs (RFLP) have shown significant differences between the two subspecies, but have not defined clearly the criteria for identifying the human-infective sub-species isolated from animal reservoirs or vectors. However, these methods are time consuming, laborious and inappropriate for the field situation (Gibson, 1989; Hide *et al.,* 1990, 1994, 1998). Therefore, the search for techniques to distinguish *T. b. rhodesiense* from *T. b. brucei* and hence its human infectivity is important.

T. b. brucei causes nagana in cattle but is not pathogenic in humans because this sub-species is lyzed by high-density lipoproteins (HDL) present in human serum. It is thought that *T. b. rhodesiense* evolved from a *T. b. brucei*-like ancestor and expresses a defence protein that ablates the anti-trypanosomal activity of human HDL. The ability of *T. b. rhodesiense* and *T. b. gambiense* to be resistant to normal human serum (NHS) enabled them to parasitize humans and cause sleeping sickness. The mechanism of resistance to NHS is still a subject for debate but previously it was believed to be due to defect in the uptake of HDL factor (Hager & Hajduk, 1997).

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This search has been nearly concluded by the discovery of the mechanism of human serum resistance in *T. b. rhodesiense.* A single gene, known as serum resistance associated (SRA) gene has been found to confer resistance to the innate trypanolytic factor (TLF) found in normal human serum. This gene allows *T.b brucei* to survive the exposure to normal human serum (Xong *et al.,* 1998). In long, slender bloodstream forms of *T. b. rhodesiense*, the expression of SRA allows neutralization of APOL1 in the lysosome. This mechanism of resistance considerably differs from the previously proposed mechanism, the selective inhibition of endocytosis of the trypanolytic factor.

The SRA gene was isolated for the first time from *T. b. rhodesiense* isolate from Uganda (De Geef *et al.*, 1989). The SRA gene is transcribed from one of the multiple telomeric loci where variant surface glycoprotein (VSG) genes are expressed (VSG expression sites). This expression site is selected when trypanosomes are grown in presence of NHS (Van Xong *et al*., 1998) The product of SRA is a atypical VSG of shorter than average length being 410 amino acids instead of approximately 490 (Vanhamme *et al.*, 2003).

The human SRA gene has been found in all *T. b. rhodesiense* isolates examined from sleeping sickness foci throughout East Africa, but not in *T. b. brucei* or any other trypanosomes of subgenus *Trypanozoon*, including *T. b. gambiense* (De Greef *et al.,* 1992; Welburn *et al.,* 2001; Radwanska *et al.,* 2002; Gibson *et al.,* 2002). This suggests that *T. b. gambiense* resists lysis through a different mechanism. Indeed *T. b. gambiense* and *T. b. rhodesiense* appear to differ in their mechanism of resistance to normal human serum. In contrast to *T. b. brucei*, the subspecies *T. b. gambiense* and *T. b. rhodesiense* escape the trypanolytic activity of human serum and cause sleeping sickness pathology. *T. b. gambiense* is permanently resistant to human serum whereas *T. b. rhodesiense* loses resistance after being isolated from humans and transferred to other animals (Hawking, 1977).

Recent advances in this field of research include a breakthrough in the diagnosis of sleeping sickness, for which the presence of SRA has proved to be a reliable marker of infection with *T. b. rhodesiense* (Gibson 2005; Gibson *et al,* 2002; Welburn *et al.,* 2001). This marker can also be used to distinguish *T. b. rhodesiense* from *T. b. gambiense*.

In this study, the SRA characterization was undertaken because the study area in western Tanzania is home to thousands of refugees from the highly *T. b. gambiense* endemic country, the Democratic Republic of Congo. It was thought that natural movement of people and influx of large number of refugees could have brought *T. b. gambiense* into western Tanzania, and also some of the isolates, both drug resistant and sensitive, identified probably could be cases of T. *b. gambiense* and not *T. rhodesiense.* The SRA marker was also used to characterize geographical origin of the isolates because this has migration implications that influence spread of drug resistant strains in the region.

Materials and Methods

Study areas

The study area included Kibondo, Kasulu and Urambo Districts in western Tanzania. The study area has been described in detail by Malele *et al*. (2006). Purposeful sampling method was used to select Kibondo, Kasulu and Urambo, because of high human African trypanosomiasis reporting cases for the past five years (Ministry of Health, unpubl.).

Isolation of trypanosomes

During the survey, people suspected to be infected with trypanosomes based on clinical observations were examined by blood smear and haematocrit centrifugation technique (HCT) in order to confirm the infection. Blood samples (2ml) were collected from confirmed sleeping sickness cases by venipuncture under the supervision of a medical doctor. Then, the patients were referred to hospital for treatment according to the stage of their illness. Each blood sample collected was divided into two portions, which were cryopreserved in liquid nitrogen. One portion was used for propagation of the isolates to the mice and the other was kept for future references.

DNA extraction from T. b. rhodesiense isolates

Genomic DNA was extracted using a commercial kit (Puregene DNA isolation kit D-7000A, Gentra Systems, Minneapolis, USA) following the manufacturer‘s instructions with minor modifications. A total of 500ml of blood was mixed with 1500ml of RBC lysis solution and incubated at room temperature for 5 minutes. The mixture was then span at 13000g for 2 minutes and the supernatant discarded. The pellet was resuspended in about 50ml of residual fluid, which was mixed with 250ml cell lysis solution containing proteinase K (100mg/ml), and incubated at 55^{o}C for 1 hour. RNAse (29mg/ml) was then added and the mixture incubated at 37^{o}C for another 45 minutes.

Protein was then isolated by addition of 200ml of protein precipitation solution, and incubated on ice for 5 minutes, followed by micro-centrifugation for 5 minutes at maximum speed. From that supernatant, DNA was precipitated by addition of 600 ml isopropanol, and the pellet was washed with 70% ethanol. The dry pellet was finally dissolved in 20ml of the DNA hydration solution included in the kit and allowed to rehydrate at 65°C for 1 hour.

PCR analysis for* T. brucei *subgroup

Primers used to amplify species specific DNA targets for *T. brucei* subgroup from isolates were TBR 1 and 2 with sequence TBR 1 5'-CGA ATG AAT AAT AAA CAA TGC GCA GT-3' and TBR2 5'-AGA ACC ATT TAT TAG CTT TGT TGC-3' (Artama *et al.,* 1992). The concentration of the primers was 0.4μM. Standard PCR amplifications were carried out in 25μl reactions mixtures containing the final concentrations, 10 mM TrisHCL pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μm of each of the 4 deoxynucleoside triphosphates and 1 unit of *RED Taq* DNA polymerase (Sigma). The amplification conditions were (30 cycles) 94°C for 30 sec, 60°C for 60 sec and 72°C for 30 sec. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and visualized under ultraviolet light.

SRA amplification from genomic DNA

PCR primers for SRA amplification were based on the DNA sequence of the SRA gene and its homologues to Kenyan *T. b. rhodesiense* isolates (EMBL accession number AF097331). Primers B537 (forward) (5'-CCA TGG CCT TTG ACG AAG AGC CCG-3') and B538 (reverse) (5'-CTC GAG TTT GCT TTT CTG TAT TTT TCC T) at 2'μM were complimentary to the 5' and 3' ends of the published SRA gene (accession no. AF097331). These primers were used to screen all trypanosome genomic DNA from 28 isolates for presence of SRA fragment by PCR (Welburn *et al.,* 2001). The amplification condition was 30 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 90 sec. Reaction volumes (25μl) which contained 1 U HotStarTaq DNA polymerase (Qiagen) supplemented with 1 U *Pfu* polymerase (Promega) and 4 mmol/L $MgCl_2$ were used. In each experiment positive controls for *T. b. brucei, T. b. gambiense* and *T b. rhodesiense* were used.

Screening for northern and southern SRA gene variants

The primers were used to screen trypanosome genomic DNA in order to identify the category (northern or southern) of SRA gene of the isolates; forward SRA H: (5'-GTACCTTGGCGCGCTCC CTGG-3') and reverse SRAJ :(5'-GTA CCT TGGCGCGCT CGCGCTG-3') (Gibson *et al.,* 2002). PCR conditions for this amplification were denaturations at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec.

Ethics declaration

This study was approved by Medical Research Coordination Committee of the National Institute for Medical Research, Tanzania. The free and informed consent of all patients who participated in this study or their legal guardians was obtained before commencement of the study

Results

Confirmation of* T. brucei *subgroup

All isolates were first confirmed as *T. brucei* subgroup by PCR analysis (Figure 1) using *Trypanozoon* primers TBR1and TBR2 before screening them for the presence of SRA gene. Since the expected band of size of 177bp was present in all, isolates screened were confirmed to belong to *T. brucei* subgroup.

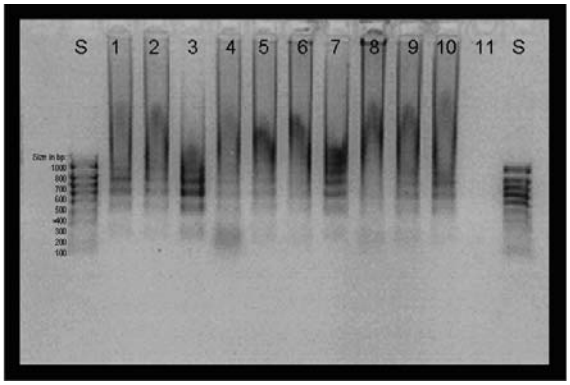

Figure 1: PCR for the confirmation of *T. brucei* subgroup of the isolates studied. Representative samples 1-10 were screened by PCR using Trypanozoon primers, TBR1 and TBR2. Lane 11 corresponds to a negative control. Lane S contains the 100 bp marker (Bioline-UK). Lane 1-10 represents isolates TMRS 3(9), TMRS 10(6), TMRS 11(2), TMRS 12 (2), TMRS 13 (2), TMRS 4 (1), TMRS 1 (13), TMRS 2 (11), KETRI 1989 and KETRI 2653, respectively. The presence of expected PCR product s of 177bp or multiples thereof confirms the isolates as *T. brucei* subgroup

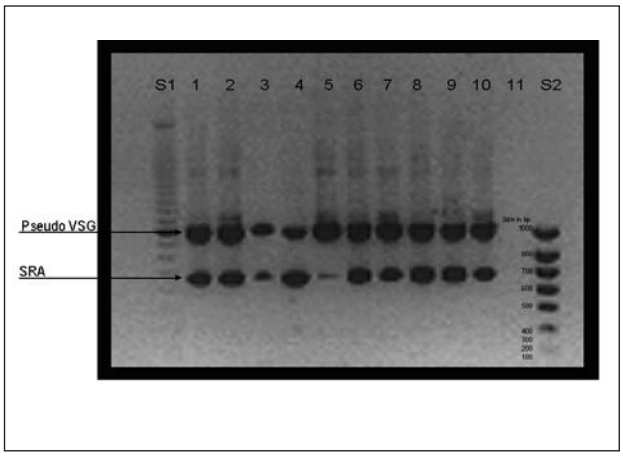

Figure 2: PCR for the identification of SRA gene among the isolates studied. Isolates were screened by PCR using primers B537 and B538 to amplify SRA gene. Lane S1 contains Hyperladder 1. Only representative isolates are shown in Lane 1-10, namely**,** TMRS 3(9), TMRS 10(6), TMRS 11(2), TMRS 12(4), TMRS 13(2), TMRS 4(1), TMRS 1(13), TMRS 2(11), KETRI 1989 and KETRI 2653 respectively. Lane 11 contains negative control and Lane S2 contains 1000bp marker. The presence of expected PCR product of 670bp or multiples thereof confirms the presence of SRA gene.

Amplification of SRA gene

The SRA fragment of approximately 670bp (Figure 2) was amplified from all 26 *T. b. rhodesiense* isolates including 2 reference isolates from Kenya. Thus the SRA gene was present in both drug susceptible and drug-resistant *T. b. rhodesiense* isolates (Table 1).

Screening for the northern and southern SRA gene variants

To determine whether they belong to northern or southern origin these isolates were screened by PCR using SRA primers (SRA H and SRA J). A prominent band of 3.9kb, was amplified from all *T. b. rhodesiense* isolates from Tanzania except two isolates probably due to technical error. The 3.9kb band was also amplified from reference strains KETRI 1989 and KETRI 2356 from Kenya. No band of 3.5kb, which is a marker for southern variant, was amplified from these isolates. All the 24 isolates from Tanzania had the northern variant of the SRA gene (Table 1). The SRA gene was specific for *T. b. rhodesiense* and could not be amplified from *T .b. gambiense* and *T. b. brucei*.

Table 1: Human SRA gene amplification from T. b. rhodesiense genomic DNA

Isolate	Origin	Year	Drug sensitivity status*	SRA**	Category***
TMRS 10 (6)	Kasulu	1999	R IS(1mg)	+	N
TMRS 11 (2)	Kasulu	2000	R M (5mg); D (14mg)	+	BA
TMRS 12 (4)	Kasulu	1999	R M (5 and 10mg); D(14 and28mg)	+	N
TMRS 13 (2)	Kasulu	1999	S	+	N
TMRS 15 (6)	Kibondo	1999	S	+	N
TMRS 7 (2)	Kibondo	1999	S	+	N
TMRS 3 (2)	Kibondo	1999	S	+	N
TMRS 3 (3)	Kibondo	1999	S	+	N
TMRS 3 (11)	Kibondo	2002	R M (5mg)	+	N
TMRS 3 (6)	Kibondo	2002	S	+	N
TMRS 4 (1)	Urambo	2002	R SU (5mg)	+	N
TMRS 2 (2)	Urambo	2002	S	+	N
TMRS 1 (13)	Urambo	2002	R IS (1mg)	+	N
TMRS 5 (1)	Tabora	2000	S	+	N
TMRS 3 (7)	Kibondo	2000	S	+	N
TMRS 9 (5)	Kasulu	2002	S	+	N
TMRS 2(11)	Urambo	2002	R SU (5mg); IS (1mg)	+	N
TMRS 8 (13)	Kasulu	2001	S	+	N
TMRS 3 (9)	Kibondo	2000	S	+	BA
TMRS 10 (3)	Kasulu	2000	S	+	N
TMRS 10 (4)	Kasulu	2000	S	+	N
TMRS 11 (3)	Kasulu	2000	S	+	N
TMRS 12 (2)	Kasulu	2000	S	+	N
TMRS 1 (2)	Urambo	2001	S	+	N
TMRS 3 (12)	Kibondo	2002	S	+	N
TMRS 12 (3)	Kasulu	1999	S	+	N
KETRI 1989	Kenya	1989	R SU and D	+	N
KETRI 2356	Kenya	1997	R M	+	N

*Drug sensitivity status is given as resistant (R) or sensitive (S).
M=Melarsoprol, D= Diminazene, SU= Suramin, IS= Isometamidium; BA= Expected but not amplified
Dosage of drug at which the isolate is resistant is given in brackets.
** The presence of SRA gene is indicated by + signs; *** All isolates were of the Northern (N) variant except two isolates which could not be classified since no band was amplified from them.

Discussion

Several studies have shown that *T. b. rhodesiense* and *T. b. gambiense* are able to infect humans due to their resistance to the cytotoxic action of normal human serum. A subfraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I, apolipoprotein L-I, and haptoglobin-related protein is toxic to *T. b. brucei* but not the human sleeping sickness parasite *T. b. rhodesiense* and *T. b. gambiense* (Faulkner *et al.,* 2006; Pays *et al.,* 2006*).* These two sub-species are largely indistinguishable from *T. b. brucei* except for their resistance to the cytotoxic action of human serum and their ability to infect humans. Therefore, the defining phenotypic trait of human sleeping sickness trypanosomes is their resistance to TLF-mediated lysis. The human serum resistance associated (SRA) gene first isolated from a Ugandan strain of *T. b. rhodesiense* has been shown to be capable by itself of conferring resistance to antitrypanosomal activity of human HDL and the trait of human infectivity on *T.b. brucei* by transfection (Gibson *et al.,* 2001).

To directly investigate this possibility, a study was carried out elsewhere for *in vitro* selection to generate human HDL-resistant *T. b. brucei*. The results showed that conversion of *T. b. brucei* from human HDL sensitive to resistant correlates with changes in the expression of the variant surface glycoprotein (VSG) and abolished uptake of the cytotoxic human HDLs (Faulkner *et al.,* 2006). These findings demonstrate that resistance to human HDLs can be acquired by *T. b. brucei.* Furthermore *T. b. rhodesiense* is likely to have arisen as a clone of *T. b. brucei* that differs mainly or solely by its ability to express SRA on selection in human serum. This gene has also been identified in several other isolates of *T.b. rhodesiense*, but not in *T. b. brucei* or any other trypanosomes of subgenus *Trypanozoon*, including another human infective *T. b. gambiense*. This gene has been found in all *T. b. rhodesiense* isolates examined from sleeping sickness foci in Tanzania confirming that it is found throughout East Africa (Ethiopia, Uganda, Kenya, Rwanda, and Zambia).

The 26 stocks of *T. b. rhodesiense* from patients in 3 districts of Tanzania were examined for the presence of the SRA gene and gave an amplified fragment of the expected size confirming that all these isolates were *T. b. rhodesiense*. Although the geographic distributions of *T. b. gambiense* and *T. b. rhodesiense* are clearly localized to west/central Africa and eastern Africa, respectively, natural movement of people and recent influx of large number of refugees into Tanzania from the Democratic Republic of Congo, could have brought *T. b. gambiense* in western Tanzania. In the Democratic Republic of Congo, the reported prevalence of *T. b. gambiense* sleeping sickness is up to 80% in some foci (Van Nieuwenhove *et al.*, 2001). The overlap in distribution of both of these pathogenic sub-species can result in erroneous diagnoses since both trypanosome sub-species are morphologically identical, and the currently available serologic techniques have low specificity (Radwanska *et al.,* 2002). Nonetheless, since these subspecies show differential drug sensitivities, a correct differential diagnosis between *T. b. gambiense* and *T. b. rhodesiense* is essential for unambiguous diagnosis of drug resistance. Therefore, the development of a simple molecular technique such as the SRA gene-based PCR may be essential for the correct diagnosis of resistance attributable to *T. b. rhodesiense*.

Both the susceptible and resistant *T. b. rhodesiense* isolates possessed the SRA gene suggesting that there is no correlation between drug resistance and presence of SRA gene. The lack of this kind of correlation suggests that these two phenomena arose by independent mechanisms. The drug resistance is most likely to be due to selection by drug pressure (Kibona *et al.,* 2006) and this selection does not affect the SRA gene. It is a valid objective to try to link observed differences in drug sensitivity with genetic markers such as the serum resistance. However, there have been few studies attempting to link drug resistance with other genotypes in East Africa. Matovu *et al.* (1997) carried out *in vitro* screening of the Ugandan *T. b. gambiense* and *T. b. rhodesiense* isolates, identified by human serum resistance (HSR) for susceptibility to melarsoprol, diminazene and isometamidium *in vitro* and found one *T. b rhodesiense* isolate resistant to the tested drugs. In view of that, the potential association between genetic markers and other characteristics such drug resistance or pathogenicity are areas worth to be explored.

The SRA gene cannot be used as sole marker for human infectivity in *T. brucei* sub-species because, it is absent in *T. gambiense* which is human infective. However, since a large variety of wild and domestic animals serve as reservoirs for both subspecies, SRA gene is a useful marker for the identification of trypanosomes of the *T. b. rhodesiense* subspecies that can infect humans (Gibson, 2001). Once the human infectivity of an isolate has been established then SRA gene can be used to distinguish *T. b. rhodesiense* from *T. b. gambiense.*

Although the *SRA* gene is conserved among *T. b. rhodesiense* isolates, there are two major sequence variants, designated northern and southern to reflect

geographical origin (Gibson *et al.,* 2002). This division tallies with previous clinical and molecular characterization studies indicating the existence of northern and southern strains of *T. b. rhodesiense* (Hide & Tilley*,* 2001; Ormerod, 1967; Gibson *et al.,* 1980; Macleod *et al.,* 2000). In this study a prominent band of a 3.9kb was amplified from Tanzanian isolates of *T. b. rhodesiense* genomic DNA. Similar findings have been observed in isolates from Uganda and Kenya (Gibson *et al.* 2002; Gibson & Ferris, 2003), meaning that all these isolates had the northern variant of the SRA gene. Thus the northern variant seems to cover the entire East Africa region including Tanzania, Kenya and Uganda.

Nevertheless, since the SRA gene resembles VSG genes and the extent of genetic evolution of this gene is currently unknown, one cannot exclude the existence of *T. b. rhodesiense* parasites with defective SRA variants or the existence of parasites with a modified SRA gene. Only two SRA genes have been characterized that differ from each other by a few point mutations (De Greef& Hamers, 1994). Moreover, in all cases where this was studied, the SRA gene appears to be a member of a large gene family that contains many pseudo genes; thus, the possibility exists that none of these sequences are functional (De Greef & Hamers, 1994). Recently, Radwanska *et al*. (2002) observed an interesting finding with strain TREU927/4, which is currently used as a reference *T. brucei* strain for genome sequencing. This strain was found to be resistant to lysis by NHS without expression of the SRA gene, even though SRA gene-related sequences were present.

Future studies involving large collections of field isolates are needed to confirm the reliability of the SRA gene PCR in identifying *T. b. rhodesiense* as the causative agent of human trypanosomiasis in different parasite foci.

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