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PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxicircle kinetoplast DNA

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Abstract

The goal of this study was to develop a PCR approach based on the sequence of maxicircle kinetoplast DNA (kDNA) of *Trypanosoma brucei* to distinguish *T. brucei*/*T. equiperdum* from *T. evansi* and to evaluate its diagnostic use for their detection in blood samples. Primers derived from the sequence of the maxicircle kDNA of *T. brucei*, encoding the NADH dehydrogenase subunit 5 (*nad5*) gene, were used to test the PCR-amplification from *T. brucei* (including *T. b. brucei* and *T. b. rhodesiense*), *T. equiperdum*, *T. evansi, T. vivax* and *T. congolense*. A primer pair to a nuclear DNA region incorporated into a separate PCR was employed to control for the presence of amplifiable genomic DNA (representing the subgenus *Trypanozoon*) in each sample subjected to the PCR. Products of ~395 bp were amplified from all *T. brucei* and *T. equiperdum* samples tested using the *nad5*-PCR, but not from *T. evansi* DNA samples or any of the control samples representing *T. vivax*, *T. congolense*, or host. The current PCR approach allows the rapid differentiation of *T. brucei*/*T.equiperdum* from *T. evansi* and can detect the equivalent of 20–25 cells of *T. brucei* or *T. equiperdum* in purified genomic DNA or infected blood samples.

Keywords: *Trypanosoma brucei*; *T. equiperdum*; *T. evansi*; Maxicircle; Kinetoplast (k) DNA; PCR

1. Introduction

The subgenus *Trypanozoon* includes three species, namely *Trypanosoma brucei*, *T. equiperdum* and *T. evansi*. *Trypanosoma brucei* is distributed in Sub-Saharan Africa and employs the tsetse fly as a vector. Within this latter species are three subspecies, *T. brucei brucei* which causes the disease Nagana in domesticated and various wild animals, and *T. b. rhodesiense* and *T. b. gambiense* which both cause distinct clinical forms of sleeping sickness in humans [1]. *Trypanosoma equiperdum* causes the disease Dourine exclusively in equines, and *T. evansi* causes Surra predominantly in livestock but also in other mammals including humans [1–3]. While small numbers of new cases of Dourine have been reported from countries, such as China, Kazakhstan, Kyrgyzstan, Pakistan, Ethiopia, Botswana, Namibia, South Africa, Brazil, Italy and Germany in the past decades, Surra is relevant in areas including Northeast Africa, Asia and South America [1]. The bloodstream forms of the three species of *Trypanosoma* are morphologically indistinguishable, except for the short, stumpy form of *T. brucei* [4].

Different approaches have been employed to detect *T. brucei*, *T. equiperdum* or/and *T. evansi* infections, including (a) parasitological ones, such as haematocrit

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centrifugation [5], mini-anion exchange centrifugation technique (mAECT) [6] and xenodiagnosis [7]; (b) serological/immunological ones, including the indirect immunofluorescence antibody test (IFAT) [8], enzyme-linked immunosorbent assay (ELISA) [9], latex agglutination test [10], card agglutination test for trypanosomiasis (CATT) [11] and the immune trypanolysis test [12]; and (c) molecular techniques based on the use of DNA hybridization and the PCR (reviewed in [13]). Given the low sensitivity and/or specificity of some parasitological and serological methods, a wide range of molecular approaches have found increased application to the diagnosis of trypanosomiases and/or the characterization of the causative agents. Some conventional DNA techniques, such as restriction fragment length polymorphism (RFLP) analysis [14], genome fingerprinting [15], analysis of repetitive DNA [16] and kinetoplast DNA (kDNA) [17] have been employed. Also, PCR-based approaches, such as minisatellite DNA analysis [18–20], amplified fragment length polymorphism (AFLP) [21,22], multiplex-endonuclease genotyping (MEGA) [23,24], mobile genetic elements (MGE)–PCR [13], simple sequence repeat (SSR)–PCR [13] and random amplification of polymorphic DNA (RAPD) [13,24–26] have been utilized for the analysis of the genetic variation within and among species of *Trypanosoma*. Recently, fluorescence in situ hybridization (FISH) with peptide nucleic acid (PNA) probes [27] and loop-mediated isothermal amplification [28] have been used. In spite of the application of these techniques, it has not yet been possible to unequivocally distinguish genetically between *T. brucei*, *T. equiperdum* and *T. evansi*. Although some primers for *Trypanozoon* members have been reported [29–31], the development of a PCR test which can differentiate reliably among species within this subgenus remains a challenge. Ventura et al. [32] developed a PCR for the detection of *T. evansi* based on a RAPD fragment, but the specificity of this PCR seems uncertain, since only a small number of samples were tested. Recently, Claes et al. [33] reported a PCR, based on the RoTat 1.2 variable surface glycoprotein (VSG) cDNA sequence, for the diagnosis of *T. evansi* infection, but it cannot distinguish *T. evansi* from *T. equiperdum*, because this VSG gene is also present in the latter species [34]. As human infection with *T. evansi* may occur in India [2] and Sri Lanka (W.C. Gibson, pers. comm.) and it is not known whether *T. evansi* infects humans in other endemic regions, the development of a simple and reliable molecular method to differentiate *T. evansi* from *T. brucei* is needed.

The members of the order Kinetoplastida contain a kinetoplast (k) DNA network, consisting of tens of maxicircles and thousands of minicircles [35,36]. The major difference at the molecular level among *T. brucei*, *T. equiperdum* and *T. evansi* is the absence of maxicircle kDNA from the latter species and a partial deletion of maxicircle kDNA from some *T. equiperdum* strains [37,38]. Masiga and Gibson [39] developed a kDNA probe based on minicircle kDNA sequence to detect *T. evansi*, but their method cannot be used to detect dyskinetoplastic (dk) strains of *T. evansi* and cannot be used to distinguish *T. evansi* from *T. equiperdum* and/or *T. brucei*. In the present study, we report a PCR approach utilizing specific primers to a sequence within the maxicircle kDNA of *T. brucei* and a set of control primers for the detection of *T. brucei* and *T. equiperdum* and their differentiation from *T. evansi*.

2. Materials and methods

2.1. Trypanosomes and DNA extraction

Nine *T. brucei* (including seven *T. b. brucei* and two *T. b. rhodesiense*), five *T. equiperdum* and ten *T. evansi* strains were used in this study, as well as strains of *T. vivax* and *T. congolense* as controls (Table 1). Trypanosomes were propagated in mice and purified from the infected mice blood using diethylaminoethyl (DEAE) cellulose (DE-52, Whatman Biochemical) [40]. DNA was released from the parasites by proteinase K digestion, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol [41]. Pellets were suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and the DNA amounts were estimated using a spectrophotometer. The quality of the genomic DNA was examined in ethidium bromide-stained agarose (0.8% w/v) gels. The genomic DNA samples from various hosts [i.e., water buffalo (*Bubalus bubalis*), horse (*Equus caballus*) and mouse (BALB/c strain)] were also extracted in the same manner.

2.2. DNA extracted directly from blood samples

Trypanosome-containing blood samples (1 ml) were collected in tubes containing 0.1 ml heparin (6250 U/ml). Samples were processed as described previously [42]. Briefly, 500 μl of saponin lysis buffer (0.22% NaCl, 0.015% saponin, 1 mM EDTA) were added to 50 μl of blood, and the mixture was then centrifuged at 10,000*g* for 1 min at 4 °C. Pellets were washed three times with the same volume of lysis buffer, resuspended in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.0, 0.5% Tween 20, 100 μg of proteinase K per ml), and incubated at 56 °C for 1 h. After an incubation of 10 min at 95 °C, 5 μl of the supernatant were used directly in the PCR.

2.3. Primers, enzymatic amplification, electrophoresis, sequencing and analysis

For the *nad*5-PCR, oligonucleotide primers were designed based on the sequence of the NADH dehydrogenase subunit 5 (*nad*5) gene (GeneBank accession number M14820) of the maxicircle kDNA of *T. brucei* pTKHR strain (see Fig. 1). The sequences of the forward primer ND5-F (5′-TGGGTTTATATCAGGTTCATTTATG-3′) and reverse primer ND5-R (5′-CCCTAATAATCTCA-TCCGCAGTACG-3′) were employed to amplify a 395 bp

Table 1
The origin of samples of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi* (subgenus *Trypanozoon*), *T. vivax* and *T. congolense* (controls) used in this study

Sample no.	Derivative designation	Species	Natural host	Place of isolation	Year of isolation
1	STIB 215	*T. brucei*	Lion	Tanzania	1971
2	STIB 247-H	*T. brucei*	Hartebeest	Tanzania	1971
3	STIB 777	*T. brucei*	Tsetse	Uganda	1971
4	STIB 920	*T. brucei*	Hartebeest	Tanzania	1971
5	STIB 940	*T. brucei*	Bovine	Somalia	1985
6	B 8/18	*T. brucei*	Pig	Nigeria	1962
7	GVR 35	*T. brucei*	Wildebeest	Tanzania	1966
8	AnTat 12.1	*T. brucei*	Human	Rwanda	1971
9	AnTat 25.1	*T. brucei*	Human	Rwanda	1971
10	OVI	*T. equiperdum*	Horse	South Africa	1975
11	BoTat 1.1	*T. equiperdum*	Horse	Morocco	1923
12	STIB 818	*T. equiperdum*	Horse	China	1979
13	STIB 841	*T. equiperdum*	na	South Africa	na
14	STIB 842	*T. equiperdum*	na	na	na
15	STIB 806	*T. evansi*	Water buffalo	China	1983
16	STIB 810	*T. evansi*	Water buffalo	China	1985
17	STIB 817	*T. evansi*	Mule	China	1964
18	RoTat 1.2	*T. evansi*	Water buffalo	Indonesia	1982
19	Merzoga 56	*T. evansi*	Camel	Morocco	1998
20	Zagora I.17	*T. evansi*	Camel	Morocco	1997
21	Colombia	*T. evansi*	Horse	Columbia	1973
22	CAN 86K	*T. evansi*	Dog	Brazil	1986
23	E110	*T. evansi*	Capybara	Brazil	na
24	2479	*T. evansi*	Camel	Kenya	1980
25	Desowitz	*T. vivax*	na	Nigeria	na
26	Y58	*T. vivax*	na	Nigeria	na
27	TC13	*T. congolense*	na	na	na

na: Information not available .

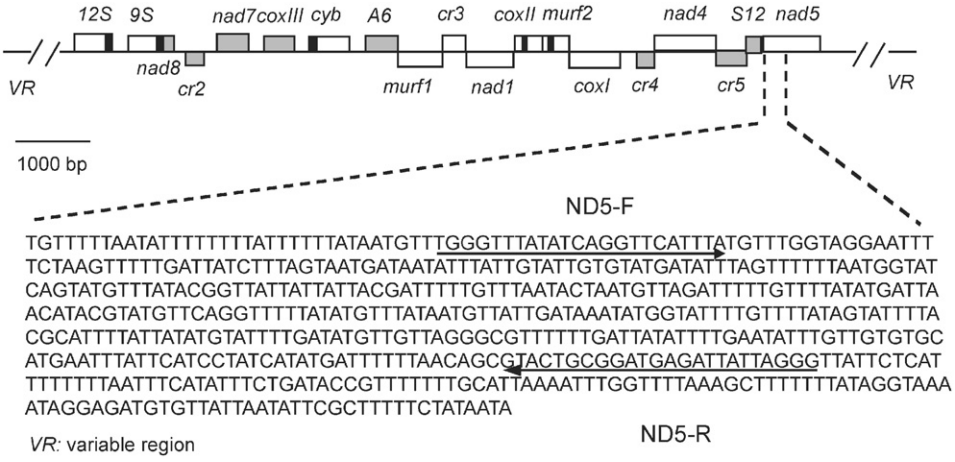

Fig. 1. Schematic representation of the genes in the maxicircle kDNA of *Trypanosoma brucei*. The approximate locations of the primers in the *nad*5 gene are indicated. The predicted size of the amplified product was 395 bp.

region of the *nad*5 gene [43]. For the *tbr*-PCR, primers TBR1 (5′-GAATATTAAACAATGCGCAG-3′) and TBR2 (5′-CCATTTATTAGCTTTGTTCG-3′) designed specifically to the (177 bp) repeats (in the nuclear genome) present in all three species within the subgenus *Trypanozoon* [44] were employed in separate control reactions to demonstrate the presence of genomic DNA in each sample representing this subgenus.

The PCR was conducted in a total volume of 50 μl using 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each of the four deoxynucleoside triphosphates (dNTPs), 1.5 U of *Taq* DNA polymerase (BBI, Canada) and 0.2 μM of each primer. For test samples, 100 ng of genomic DNA (for the *nad*5-PCR), 10 ng (for the *tbr*-PCR) or 5 μl of blood supernatant were included in the PCR. For the negative controls, H_2O was added instead of DNA. For

the *nad5*-PCR, the cycling conditions were: an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension for 10 min at 72 °C. For the *tbr*-PCR, the cycling conditions were an initial denaturation for 5 min at 95 °C, followed by 30 cycles of 45 s at 94 °C, 1 min at 60 °C and 30 s at 72 °C, and a final extension for 5 min at 72 °C. The amplicons were subjected to electrophoresis in ethidium bromide-stained agarose (1.5% w/v) gels, which were photographed using an electronic documentation system (Biostep, Germany).

Selected *nad5*-PCR amplicons were sequenced in both directions (using BigDye® chemistry, Applied Biosystems), employing the same primers as for the primary PCR. The alignment of sequences from the six amplicons relative to the sequence of *T. brucei* pTKHR strain (accession no. M14820) was carried out using MEGA 3.0 software [45].

3. Results and discussion

Fig. 2 shows the results achieved using the *nad5*-PCR. A single band of ~395 bp was detected for each DNA sample representing *T. brucei* ($n = 9$) and *T. equiperdum* ($n = 5$). No amplicons were produced for any of the *T. evansi* ($n = 10$), *T. vivax* ($n = 2$) or *T. congolense* ($n = 1$) samples or from the DNA control samples from water buffalo, horse or mouse. Six amplicons representing lanes 3, 5, 7 and 10–12 (Fig. 2) were selected for sequencing and the sequence data were compared with a *nad5* gene sequence from *T. brucei* pTKHR strain (accession no. M14820) [46] available in the GeneBank database. Sequence variation of ⩽3% (over 360 nucleotides) was measured among the six sequences (data not shown). For the *tbr*-PCR, an amplicon of ~177 bp was amplified from all 24 DNA samples representing the subgenus *Trypanozoon* (thus demonstrating amplifiable DNA in each case) but not from *T. vivax*, *T. congolense* or host DNA controls (Fig. 2).

To establish the threshold of detection in the *nad5*-PCR, a dilution series (ten-fold) of DNA corresponding to 2×10^5–0.2 trypomastigotes per PCR was tested. Similarly, a dilution series (ten-fold) of infected mouse blood (containing 10^6 trypomastigotes of *T. brucei* STIB 940 strain per ml) corresponding to 2.5×10^3–0.025 trypomastigotes was performed and tested in the PCR. The *nad5*-PCR was able to detect ⩾20 trypanosomes using purified DNA and ⩾25 using infected blood (see Fig. 3).

Based on the findings of the present study, the maxicircle kDNA is considered to provide a useful genetic marker to distinguish *T. brucei*/*T. equiperdum* from *T. evansi*, because of its presence in the former species pair and absence from the latter species. The kDNA corresponds to the mitochondrial DNA in other eukaryotes but is complex structurally [47]. In *T. brucei*, the kDNA network generally consists of 50–100 maxicircles, which encode the ribosomal RNAs and proteins necessary for mitochondrial function, and 5000-10,000 minicircles which are heterogeneous in sequence and encode the "guide RNAs" employed for editing maxicircle transcripts [48,49]. *Trypanosoma equiperdum* and *T. evansi,* however, are morphologically indistinguishable from *T. brucei* and they do not undergo cyclical development [50]. The kDNA of the former two species contains a single, major class of "homogenous" minicircles [35,51–53]. By comparison with *T. brucei*, the kDNA of *T. evansi* contains no maxicircles,

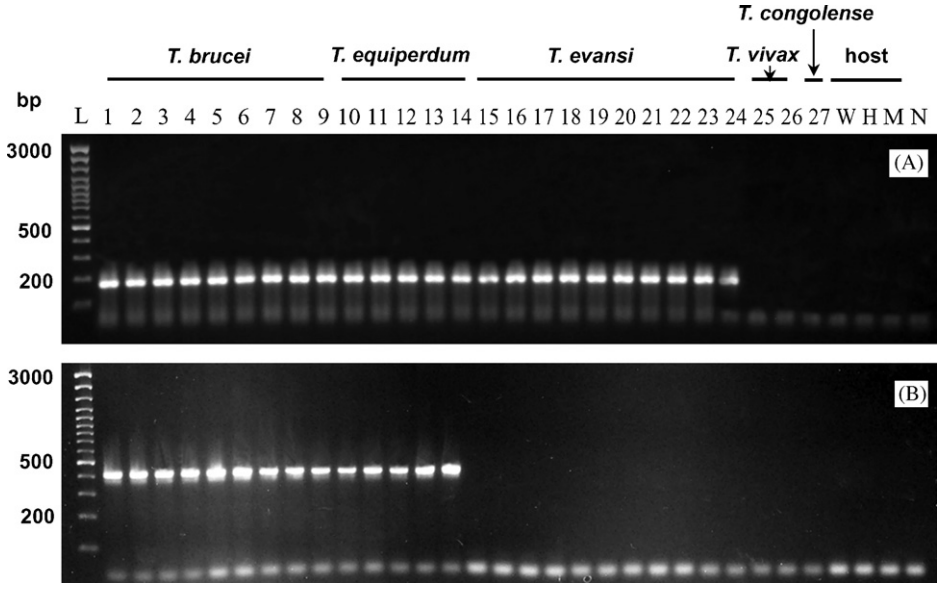

Fig. 2. PCR differentiation of *Trypanosoma brucei*/*T. equiperdum* from *T. evansi*. Agarose gel displaying amplicons produced from DNA using the *tbr*-PCR (A) and the *nad5*-PCR (B). The lanes 1–27 represent the same species and strains of *Trypanosoma* as listed in Table 1. Lanes W, H and M represent water buffalo (*Bubalus bubalis*), horse (*Equus caballus*) and mouse (BALB/c) (host) DNA controls, respectively. Lane L represents a 100 bp plus DNA ladder (BBI, Canada), and lane N represents a no-DNA (negative) control.

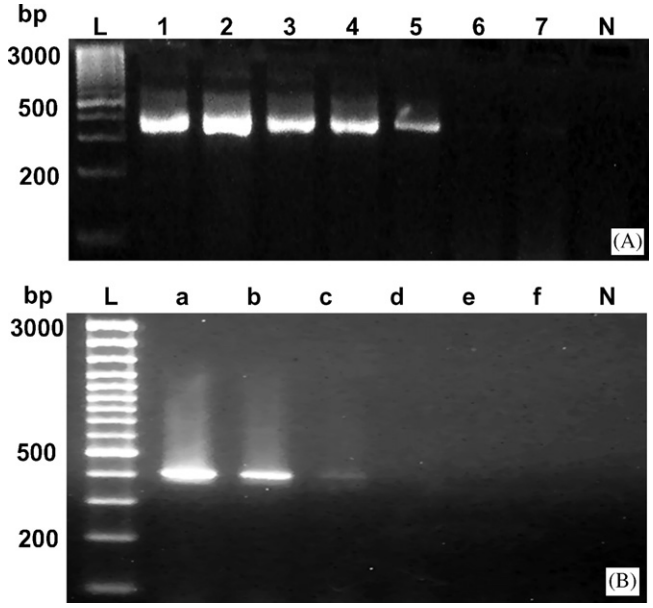

Fig. 3. Agarose gels displaying amplicons produced by PCR from a dilution series of DNA from *Trypanosoma brucei* STIB 940 (panel A) or blood samples from mice infected with the same strain (panel B). To individual PCR tubes were added an equivalent of 2×10^5, 2×10^4, 2×10^3, 2×10^2, 20, 2 cells and 0.2 cell (panel A, lanes 1–7, respectively), or 2.5×10^3, 2.5×10^2, 25, 2.5 trypanosomes, 0.25 and 0.025 trypanosome (panel B, lanes a–f, respectively). Lane L represents a 100 bp plus DNA ladder (BBI, Canada), and lane N represents a no-DNA (negative) control.

and partial deletion of maxicircles can occur in some strains of *T. equiperdum*. The maxicircles of *T. equiperdum* (Pasteur strain) are ~24.5 kb [51], being similar in size to those of *T. brucei*. However, the maxicircles of two *T. equiperdum* strains (i.e. ATCC30019 and STIB 818) are ~14 and 14.3 kb in size, respectively [17,37]. The present results demonstrate that a fragment was amplified specifically in the PCR from all members of the subgenus *Trypanozoon*, namely *T. b. brucei, T. b. gambiense, T. b. rhodesiense* and *T. equiperdum* but not from *T. evansi* using the maxicircle kDNA primers (Fig. 2). Although the specific fragment was also produced for the five strains representing *T. equiperdum,* this does not affect the outcome, as this species of *Trypanosoma* exclusively infects equines and is rarely detected in the blood stream of its host [54]. This species is localized to the capillaries of the mucous membranes of the urogenital tract. Also, *T. equiperdum* can be distinguished from *T. brucei* when cultured in vitro at 27 °C; the blood-stream forms of *T. brucei* can change from trypomastigotes to epimastigotes, which is not the case for *T. equiperdum* [38].

It has long been considered that *T. evansi* does not infect humans because the parasites can be lysed by high-density lipoprotein (HDL) in normal human serum (NHS) [55]. However, human trypanosomiasis caused by *T. evansi* has been reported in India [2]. This finding suggests that this species of trypanosome is of increasing public health concern, particularly because of its broad geographical distribution. Therefore, the present PCR approach (using two primer sets) may provide a means of assisting in the diagnosis of *T. evansi* infection, although mixed infections of *T. evansi* and *T. brucei* could not be differentiated from monospecific *T. brucei* infection.

By comparison with previous PCR tests developed to detect *T. evansi* based on minicircle kDNA [39,56], the present PCR approach seems simple and reliable. Since the number of natural occurred *T. evansi* dk-strains has been reported to be relatively high [57,58], any PCR method based on the amplification of minicircle kDNA will not work for these strains. In contrast, the number of spontaneously occurring *T. brucei* dk-strains is considered to be rare [57]. In fact, thus far, natural *T. brucei* dk-strains have not been reported, although one such strain has been induced in the laboratory using acriflavine [57].

Other PCR tests based on genomic DNA have been described previously. For instance, Ventura et al. [32] developed a PCR method for the detection of *T. evansi* based on a RAPD fragment Te664. However, Claes [59] reported that this PCR, initially thought to be *T. evansi* specific, gave positive results for some strains of *T. b. brucei* and *T. b. gambiense* and was thus not considered specific for *T. evansi*. Furthermore, based on the analysis of the RoTat 1.2 VSG gene sequence, Claes et al. [33] developed a PCR method to distinguish *T. evansi* from other species within the subgenus *Trypanozoon*. Their results showed that all *T. evansi* and *T. equiperdum* samples examined, with the exception of OVI and BoTat 1.1 strains, contained the target sequence, but this fragment was not amplified from all *T. brucei* strains tested. Thus, these authors [33] concluded that these PCR primers designed based on the RoTat 1.2 VSG gene sequence of *T. evansi* were specific for *T. evansi* strains. However, results from Ngaira et al. [34,60] revealed that the RoTat 1.2 VSG gene was absent from some *T. evansi* strains isolated from camels in Kenya. Verloo et al. [61] also demonstrated by immunofluorescence that the RoTat 1.2 variable antigen type (VAT) was not expressed during primary parasitaemia in two *T. evansi* strains from the Philippines and China. This information may cast some doubt on the specificity of this method [33]. Thus far, no false-negative or false-positive results have been detected using the 19 *T. brucei* and *T. evansi* samples tested herein. Using a greater number of samples from different geographical and host origins, the specificity (and sensitivity) of the PCR approach will continue to be monitored.

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