Running title: Molecular dipstick for sleeping sickness

A molecular dipstick test for the diagnosis of sleeping sickness

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Summary

Human African trypanosomiasis (HAT) or sleeping sickness, is a neglected disease that affects poor rural populations across sub-Saharan Africa. Confirmation of diagnosis is based on detection of parasites in either blood or lymph by microscopy. Here we present the development and the first phase evaluation of a simple and rapid test (HAT-PCR-OC) for detection of amplified *Trypanosoma brucei* DNA. PCR products are visualised on a dipstick through hybridisation with a gold conjugated probe (oligochromatography). Visualisation is straightforward and takes only 5 minutes. Controls for both the PCR reaction and for DNA migration are incorporated in the assay. The lower detection limit of the test is 5 fg of pure *T. brucei* DNA. One parasite in 180 µl of blood is still detectable. Sensitivity and specificity for *T. brucei* were calculated at 100% when tested on blood samples from 26 confirmed sleeping sickness patients, 18 negative controls (non-endemic region) and 50 negative control blood samples from endemic region. HAT-PCR-OC is a promising new tool for diagnosis of sleeping sickness in laboratory settings and the diagnostic format described here may have wider application for other infectious diseases.

Introduction

Human African trypanosomiasis (HAT) is a complex of protozoan infections, fatal if untreated, which can be caused by infection with either *Trypanosoma brucei* (*T. b.*) *gambiense* (chronic HAT) or *T. b. rhodesiense* (acute HAT). Both human infective sub-species are cyclically transmitted by tsetse flies (genus *Glossina*). HAT is a severely neglected disease, typically affecting rural populations of poor people across sub-Saharan Africa (23). Over 60 million people are at risk of contracting the disease with an estimated 50-70,000 persons newly infected annually (2). In the absence of treatment, HAT patients inevitably die after a more or less prolonged period of grave illness. Disease control for *T. b. gambiense* relies heavily on accurate diagnosis and effective treatment of patients and for *T. b. rhodesiense* on treatment of patients and of the animal reservoir (24). Classical diagnosis requires demonstration of parasites in blood or lymph which for *T. b. gambiense* infection is problematic due to extremely low parasitaemias in infected people. It is estimated that 20 to 30% of the patients remain undiagnosed by standard parasitological techniques (20). New molecular techniques based on the polymerase chain reaction (PCR) have been developed as surrogate for parasite detection. Applications of PCR for detecting *T. brucei* and its subspecies have been reported, which can be highly effective for detection of *T. b. rhodesiense* and for distinguishing *T. b. rhodesiense* from *T. b. gambiense* (7, 16, 17, 18, 22, 25). The use of these highly specific and sensitive PCR methods is however only suitable for screening for disease prevalence and for research purposes and are not commonly used to inform diagnosis of HAT (5). This is partly due to the cumbersome methods of PCR product detection. Amplicons are normally identified using UV trans-illumination after being electrophoresed in the presence of ethidium bromide (a carcinogen). Alternative methods for

PCR product detection such as real-time PCR, PCR-ELISA or mass spectrometry have been developed (3, 10, 21) but are complex, expensive, equipment, recourse and personnel hungry. There is a demand for a simplified method of amplification and product detection which would make these tools useable in the facilities available in regional sleeping sickness diagnostic laboratories.

Oligochromatography (OC) provides a simple and rapid dipstick format for detection of amplified PCR products (Coris BioConcept, Gembloux, Belgium; Patent n° WO 2004/099438A1) (12, 13, 15, 19). PCR products are visualised by hybridisation with a gold conjugated probe. This PCR product detection format takes only 5 minutes and no other equipment than a dry heating block and a pipette are needed. An internal control (IC) for the PCR reaction and a control for the chromatographic migration are incorporated in the assay.

We present here the development and phase I evaluation of a *T. brucei* specific PCR-Oligochromatography test, called HAT-PCR-OC (Human African Trypanosomiasis-Polymerase Chain Reaction-Oligochromatography). We have chosen the 18S ribosomal RNA gene (rDNA) as the target for the HAT-PCR-OC since it is a multi copy gene that contains sequences conserved within *Trypanosomatids* and species specific sequences (11).

Methods

Parasite DNA

DNA was obtained from *T. b. gambiense* (LiTat 1.3) and *T. b. rhodesiense* (STIB 382) parasites amplified in HsdCpb:WU rats (Harlan, the Netherlands). Trypanosomes were separated from the blood using di-ethyl-amino-ethyl (DEAE) chromatography (9), followed by repeated centrifugation (20 minutes, 2000 g) and sediment washes with Phosphate

Buffered Saline Glucose (PSG) (38 mM $Na_2HPO_4.2H_20$, 2 mM $NaHPO_4$, 80 mM glucose, 29 mM NaCl, pH 8.0). Trypanosome pellets were stored at –80°C. Twenty µl of trypanosome pellet (approximately 2×10^7 cells) were resuspended in 200 µl of Phosphate Buffered Saline (PBS) (8.1 mM $Na_2HPO_4.2H_2O$, 1.4 mM $NaHPO_4$, 140 mM NaCl, pH 7.4) and the DNA was extracted with the QIAamp DNA mini kit (Qiagen, Hilden, Germany), resulting in pure DNA in 200 µl TE elution buffer. The typical yield of DNA extracted from a 20 µl pellet was 150 ng/µl or 30 µg of total DNA. The extracts obtained were diluted 200 times in water and divided into aliquots of 2 ml in microcentrifuge tubes for storage at –20°C. Purified DNA from other pathogens, *i.e. Leishmania donovani*, *Trypanosoma cruzi*, *Mycobacterium tuberculosis*, *Plasmodium falciparum* and *Schistosoma mansoni* was obtained from other research groups.

Blood samples

Informed consent was obtained from patients, or their parents or guardians and from non-diseased persons. The human and animal experimentation guidelines of the Institute of Tropical Medicine (Antwerp, Belgium) were followed.

Non endemic controls

Venous blood on EDTA from 28 healthy human Belgian blood donors who had never visited a HAT endemic country (6).

Endemic controls

Venous blood on EDTA from 50 healthy human volunteers in Kinshasa, D.R. Congo.

Sleeping sickness patients

Venous blood on heparin from 26 *T. b. gambiense* patients enrolled in a clinical study performed in D. R. Congo and with confirmed presence of parasites in the blood.

Spiked blood

Blood on EDTA spiked with *T. b. gambiense* (LiTat 1.3) parasites was used throughout the development of the assay and for the estimation of its lower detection limit. Bloodstream form trypanosomes were grown in rats. At day 3 post infection, tail blood was taken and diluted in PSG and the number of parasites per ml was counted in a KOVA cell counting chamber (Hycor Biomedical Inc., California, USA). A 10-fold dilution series of parasites was made in freshly taken naive human blood, ranging from 10,000 parasites/180 µl to 1 parasite/180 µl blood. Non-spiked blood was used as negative control.

DNA extraction from blood samples

As recommended by Qiagen, 180 µl of blood were mixed with an equal volume of AS1 buffer (Qiagen, Hilden, Germany) and stored at ambient temperature until DNA extraction. Blood can be stored in this stabilising buffer for up to three months in the dark without loss of DNA quality. DNA was extracted with the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at –20°C. Briefly, DNA is adsorbed onto the silica-gel membrane in the QIAamp Spin Column, washed by two centrifugation steps and eluted in 50 µl elution buffer. 180 µl sample of whole blood will yield an estimated 3 - 12 µg of DNA. The internal control line in the oligochromatography (see below) showed the presence of inhibitory factors in the extracted DNA in some samples (1). In such cases, DNA was further purified as follows: 1/10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of ice-cold 100% ethanol were added, followed by overnight DNA precipitation at -20°C. The suspension was centrifuged at 16,100 g for 15 minutes and the

DNA resuspended in 500 µl of 70% ethanol. The suspension was again centrifuged for 10 minutes at 16,100 g and the ethanol was removed. DNA was dried in a vacuum chamber for at least 30 minutes and finally resuspended in 50 µl of water and stored at -20°C.

Primers and probes (Fig. 1)

Primers, biotinylated primers, internal control DNA and probes were synthesised by Sigma (Bornem, Belgium).

Primers

Sequences of the 18S ribosomal RNA gene (rDNA) of the *Trypanosomatidae* parasites *T. brucei gambiense* (GenBank accession number AJ009141), *T. brucei rhodesiense* (GenBank accession number AJ009142), *T. cruzi* (GenBank accession number AF303660) and *L. donovani* (GenBank accession number X07773) were aligned and primers (18S-F and 18S-R) were designed to amplify a sequence of the *Trypanosomatidae* 18S rDNA using the 'DNAman' software (Lynnon Corporation, Quebec, Canada). Primers are situated in *Trypanosomatidae* conserved sequences that surround a *T. brucei* conserved sequence. The reverse primer 18S-R was biotinylated at the 5' end.

T. brucei gold probe

The alignments described above were used to design a 17 bp DNA probe that can hybridise with a *T. brucei* specific sequence situated between the *Trypanosomatidae* conserved primer sequences. Absence of putative secondary structures within the expected PCR amplicon was checked via mfold version 3.1. (26). The probe was conjugated with gold particles using the procedure described in Patent WO 2004/099438A1 (19).

Internal control DNA

The internal control DNA (IC DNA) was constructed to be of the same length as the *T. brucei* sequence (106 bp). Its sequence is identical to the *T. brucei* sequence except for the 17 bp central part. This central part was designed to have the same GC content as the *T. brucei* 17 bp sequence (Patent WO 2004/099438A1) (19).

IC gold probe

A DNA probe was designed that can hybridise with the 17 bp IC specific sequence within the IC DNA. This IC probe was conjugated with gold particles as described above.

Migration control probe

The migration control probe is identical to the 17 bp central sequence of the IC DNA.

PCR amplification

An asymmetric PCR was designed using 4 fold more biotinylated reverse primer than forward primer. The 50 µl reaction mixture contained 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl (PCR buffer, Sigma, Bornem, Belgium), 200 µM of each deoxynucleotide triphosphate (Roche, Mannheim, Germany), 2.5 mM $MgCl_2$ (Sigma, Bornem, Belgium), 0.2 µM forward primer 18S-F, 0.8 µM biotinylated reverse primer 18S-R, 0.1 mg/ml acetylated bovine serum albumine (Promega, Madison, USA), 3.2 aM IC DNA, 1 U of HotStar *Taq* polymerase (Qiagen, Hilden, Germany) and 5 µl of sample DNA. An initial denaturation step of 94°C for 15 min to activate the HotStar *Taq* polymerase was followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 minutes. Amplification was conducted in 200 µl thin-wall PCR tubes (Abgene, Epsom, UK) in a T3 thermocycler 48 (Biometra, Göttingen, Germany).

Oligochromatography

Preparation

The Oligochromatography (OC) dipstick is constructed with polymer backing faced on each side with a lower absorbent pad which overlaps a nitrocellulose membrane (in the middle) and an upper absorbent pad (Fig. 2). The facing side represents the test for *T. brucei* and the back is the control. The lower absorbent pad at the test side is impregnated with the *T. brucei* gold probe and the lower absorbent pad at the control side is impregnated with the IC gold probe. On the membrane at the test side, a line of Neutralite avidin (Belovo SA, Bastogne, Belgium) is coated. On the membrane at the control side, two control lines are coated. A first line of Neutralite avidin (control for PCR) and a second line of a probe that is complementary to the IC gold probe (migration control). The OC conditions for the phase I evaluation were as follows. The PCR product was denaturated at 94°C for 30 seconds and kept on ice. Forty µl were mixed with an equal volume of migration buffer preheated at 55°C followed by dipping the OC dipstick into the solution.

Principle

After amplification of a sample containing *T. brucei* DNA, the PCR product solution contains both IC amplicons and *T. brucei* amplicons. During migration at 55°C through the lower absorbent pad, the solution takes up the impregnated gold probes. The *T. brucei* gold probe hybridises with the biotinylated strands of the *T. brucei* amplicons during migration. These complexes accumulate on the Neutralite avidin line at the test side resulting in a visible red line. On the back side the IC gold probe hybridises with the biotinylated strands of the IC amplicons and these complexes accumulate on the Neutralite avidin line. Unbound IC gold probe migrates further and is trapped by the complementary migration control probe

line. Both control lines determine whether the HAT-PCR-OC is valid or invalid (Fig. 3). The migration control line should always be visible to validate the test. A test is invalid when both the IC amplicon control line and the *T. brucei* test line are invisible (Fig. 3d) indicating that the PCR reaction failed possibly due to inhibitory factors in the extracted DNA. When a sample contains very high concentrations of *Trypanosomatidae* DNA, competition between this target DNA and the IC template DNA for the primers can result in an invisible IC amplicon control line combined with a strongly visible *T. brucei* test line (Fig. 3c). In the latter case, the test is considered valid.

Results

Analytical sensitivity

The detection limit of the HAT-PCR-OC was evaluated on a serial dilution of *T. b. gambiense* DNA in water. The lower detection limit was 5 fg of parasite DNA which is about 1/40 of the DNA content of one parasite (Fig. 4a). Moreover, the test was able to detect ten times less DNA compared to agarose gel electrophoresis (data not shown). The lower detection limit was also evaluated on DNA extracted from a blood sample series spiked with decreasing numbers of living *T. b. gambiense* parasites. HAT-PCR-OC was consistently able to detect one single parasite in 180 µl of blood (Fig. 4b). The assay remained negative when non-spiked control blood samples were tested.

Analytical specificity

The species specificity of the HAT-PCR-OC was assessed on purified DNA from *T. b. gambiense, T. b. rhodesiense, Leishmania donovani, Trypanosoma cruzi, Mycobacterium*

tuberculosis, *Plasmodium falciparum* and *Schistosoma mansoni*. Positive results were only obtained with the two *T. brucei* subspecies (Fig. 4c).

Diagnostic sensitivity and specificity (Fig. 5)

HAT-PCR-OC was positive with the blood samples from all 26 *T. b. gambiense* sleeping sickness patients and negative with blood samples from all 28 healthy Belgian blood donors. With the 50 endemic negative control blood samples, 47 tests were valid and negative in HAT-PCR-OC. Three test results were invalid since the IC for PCR was negative, indicating the presence of PCR inhibitory factors in the extracted DNA. Therefore, DNA of these samples was ethanol precipitated and resuspended in water. When re-tested, valid negative test results were obtained. Thus, HAT-PCR-OC results corresponded fully with the infection status of the sampled persons indicating 100% sensitivity and specificity.

Discussion

A major constraint for effective sleeping sickness control is imperfect diagnosis. Reliable and fast diagnostic tools are desperately needed. The HAT-PCR-OC test described here has been developed as a simplified molecular test for rapid and sensitive detection of *T. brucei* parasites in blood samples. HAT-PCR-OC combines both the sensitivity and specificity of PCR with the simplicity and speed of membrane chromatography (visible after 5 minutes by the naked eye). OC, in contrast to conventional amplicon detection techniques, doesn't require post-amplification preparation, neither sophisticated equipment. The data presented here show that the assay is able to detect one single parasite in a 180 µl volume of blood. When evaluated using both trypanosome positive patient and control trypanosome negative samples, HAT-PCR-OC results corresponded 100% with the infection status of the tested persons.

HAT-PCR-OC shows clear potential for implementation as a reference diagnostic test in mid level equipped laboratory facilities (clean laboratories with power and cold storage) and will be of particular value for evaluation of cure assessment during clinical trials of new drugs or drug combinations. The simple detection methodology described here may facilitate PCR application in HAT endemic countries. Further refinement of this assay will widen its application. New simple nucleic acid extraction techniques together with novel isothermal nucleic acid amplification methods such as loop-mediated isothermal amplification (LAMP) (8, 14) and nucleic acid based amplification (NASBA) (4) will mean that the constraint to field PCR (namely the need for thermocycling reactions) could be eliminated.

A further application of the HAT-PCR-OC test format would be for diagnosis of all *Trypanozoon* infections since the detection probe is specific for all *Trypanozoon* taxa

including *T. b. brucei*, *T. evansi* and *T. equiperdum* that affect cattle, buffaloes, small ruminants, camel and horses and that are responsible for severe losses in the agricultural sector. This is of particular value for *T. b. rhodesiense* HAT in which case the disease is maintained in the domestic animal reservoir (24).

A common drawback of PCR is the risk of sample contamination with PCR products leading to false positive results. When implementing HAT-PCR-OC, one should take measures to avoid this risk. The oligochromatography procedure should be performed in closed test tubes and pre- and post-PCR manipulations should be physically separated, preferably in separate lab spaces. Finally, used dipsticks should be thrown away after reading the result or should be archived in a post amplification room.

To our knowledge this is the first nucleic acid based diagnostic test in dipstick format for vector-borne diseases. The proof of principal for HAT-PCR-OC presented in this study opens perspectives for its use in laboratories within countries where sleeping sickness is endemic. The same format can be applied for other infectious diseases such as malaria, tuberculosis, leishmaniasis, Chagas' disease, schistosomiasis, *etc*. for which more simple and applicable molecular diagnostic tools are needed.

Acknowledgements

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Figure 1. Alignment of the HAT-PCR-OC target sequence within the 18S rRNA gene of the *Trypanosomatidae* parasites *Trypanosoma brucei gambiense (T.b.g.)* (GenBank accession number AJ009141), *Trypanosoma brucei rhodesiense (T.b.r.)* (GenBank accession number AJ009142), *Trypanosoma cruzi* (*T.c.*) (GenBank accession number AF303660) and *Leishmania donovani* (*L.d.*) (GenBank accession number X07773). Situation of the forward primer 18S-F, the biotin labelled reverse primer 18S-R, and the *T. brucei* (*T.b.*) detection probe. Gaps in the sequence are presented by dots.

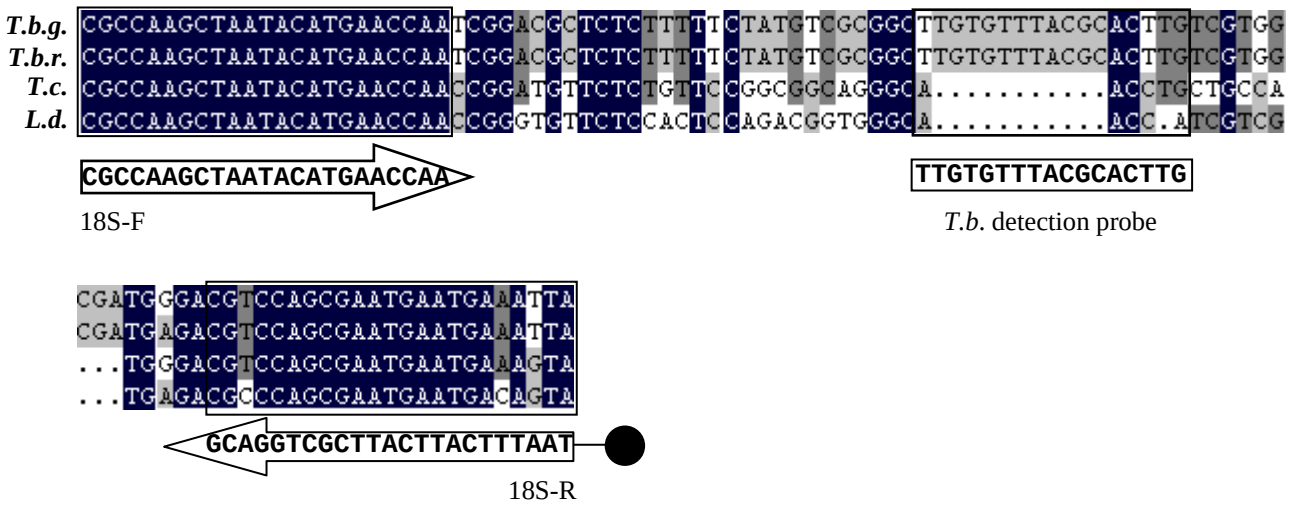


Figure 2. HAT-PCR-OC test principle

Test side. In case of a positive sample, during migration, the *T. brucei* gold probes hybridise with the *T. brucei* amplicons that will accumulate on the Neutralite avidin line on the test side of the stick.

Control side. During migration the internal control (IC) gold probes hybridise with IC amplicons that will accumulate on the Neutralite avidin line on the control side of the stick (control for PCR). The unbound IC gold probes hybrise with the complementary migration control probes (migration control).

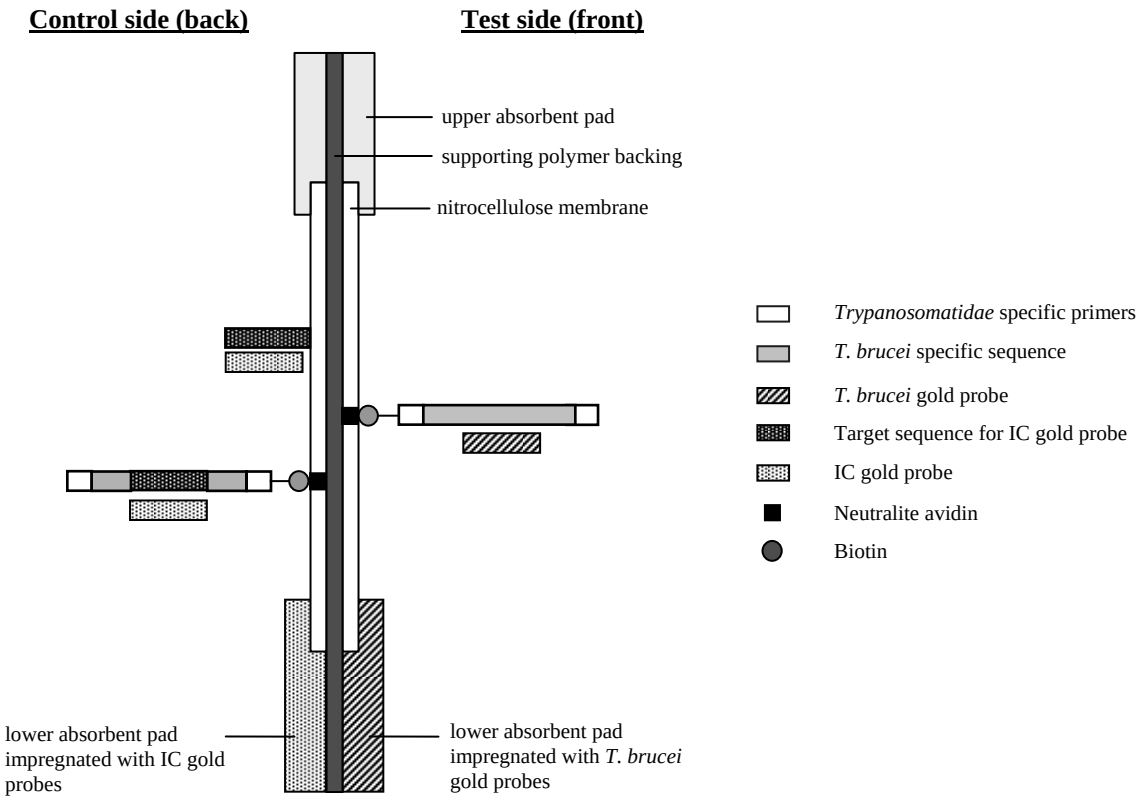


Figure 3. Possible HAT-PCR-OC test result. a. valid test, positive result, b. valid test, negative result, c. valid test, positive result of a sample with high *T. brucei* DNA content, d. invalid test

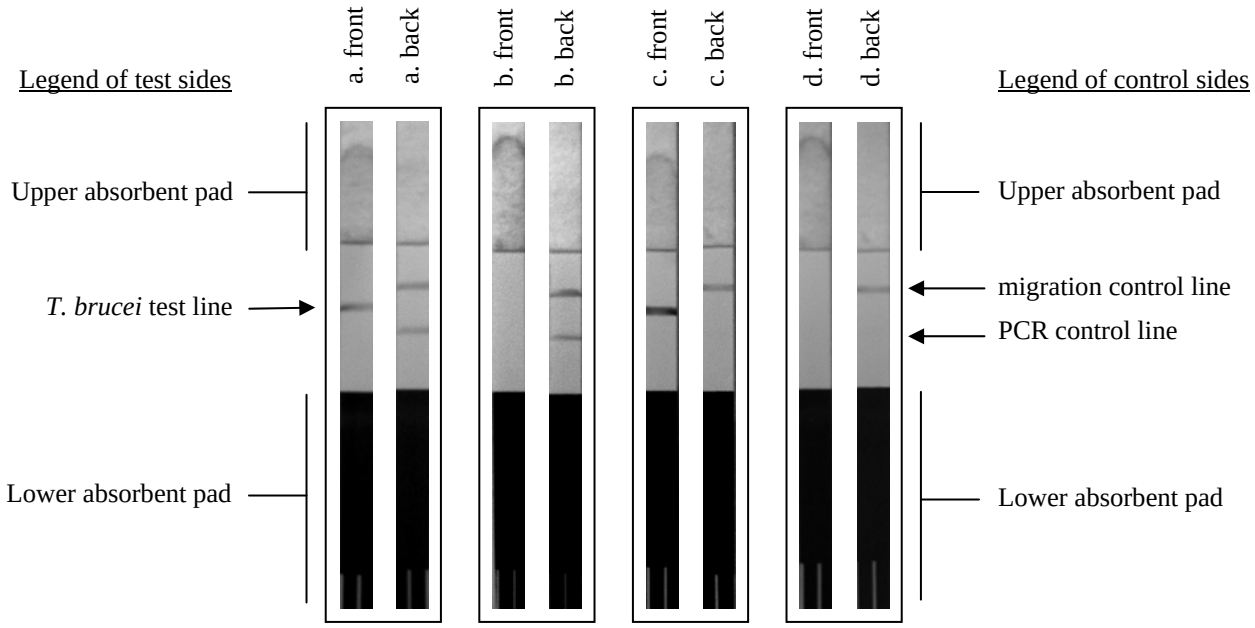


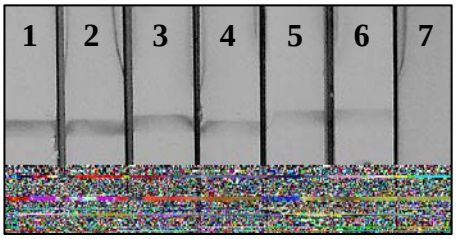
Figure 4. Analytical sensitivity and species specificity of the HAT-PCR-OC test

a. Serial dilution of *T. b. gambiense* DNA in water. Dipstick 1 - 7: 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 5 fg, 0.5 fg per PCR reaction

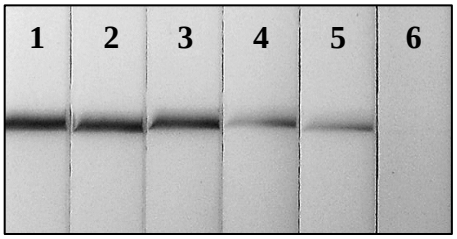
b. Serial dilution of living *T. b. gambiense* bloodstream form parasites in naïve human blood. Dipstick 1 - 6: 10,000, 1,000, 100, 10, 1, 0 parasites in 180 µl of blood.

c. HAT-PCR-OC results obtained with DNA form *T. b. gambiense* (dipstick 1), *T. b. rhodesiense* (dipstick 2), *Leishmania donovani* (dipstick 3), *Trypanosoma cruzi* (dipstick 4), *Mycobacterium tuberculosis* (dipstick 5), *Plasmodium falciparum* (dipstick 6) and *Schistosoma mansoni* (dipstick 7).

a.



b.



c.

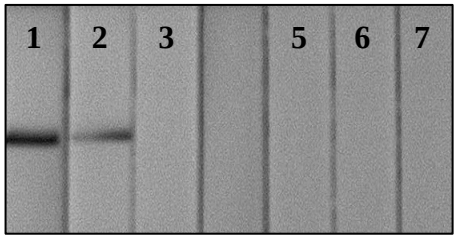


Figure 5. Diagnostic sensitivity and specificity of the HAT-PCR-OC.

a. HAT-PCR-OC results for 26 blood samples from confirmed *T. b. gambiense* sleeping sickness patients (dipstick 1 – 26) and one negative control (-).

b. HAT-PCR-OC results for one positive control (+) and for 5 blood samples from endemic negative controls (dipstick 1 – 5).