



## Development and evaluation of an ITS1 “Touchdown” PCR for assessment of drug efficacy against animal African trypanosomosis



Thao Tran<sup>a,b</sup>, Grant Napier<sup>a</sup>, Tim Rowan<sup>a</sup>, Claudia Cordel<sup>c</sup>,  
Michel Labuschagne<sup>c</sup>, Vincent Delespau<sup>b</sup>, Nick Van Reet<sup>b</sup>, Heidi Erasmus<sup>c</sup>,  
Annesca Joubert<sup>c</sup>, Philippe Büscher<sup>b,\*</sup>

<sup>a</sup> Global Alliance for Livestock Veterinary Medicines (GALVmed), Doherty Building, Pentlands Science Park, Bush Loan, Edinburgh EH26 0PZ, UK

<sup>b</sup> Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

<sup>c</sup> ClinVet International (Pty) Ltd., Uitzigweg, Bainsvlei, Bloemfontein, South Africa

### ARTICLE INFO

#### Article history:

Received 6 December 2013

Received in revised form 26 February 2014

Accepted 1 March 2014

#### Keywords:

ITS1

Touchdown PCR

*Trypanosoma congolense*

*Trypanosoma vivax*

Trypanozoon

Cattle

### ABSTRACT

Animal African trypanosomoses (AAT) are caused by flagellated protozoa of the *Trypanosoma* genus and contribute to considerable losses in animal production in Africa, Latin America and South East Asia. *Trypanosoma congolense* is considered the economically most important species. Drug resistant *T. congolense* strains present a threat to the control of AAT and have triggered research into discovery of novel trypanocides. *In vivo* assessment of trypanocidal efficacy relies on monitoring of treated animals with microscopic parasite detection methods. Since these methods have poor sensitivity, follow-up for up to 100 days after treatment is recommended to increase the chance of detecting recurrent parasitaemia waves. Molecular techniques are more amendable to high throughput processing and are generally more sensitive than microscopic detection, thus bearing the potential of shortening the 100-day follow up period. The study presents a “Touchdown” PCR targeting the internal transcribed spacer 1 of the ribosomal DNA (ITS1 TD PCR) that enables detection and discrimination of different *Trypanosoma* taxa in a single run due to variations in PCR product sizes. The assay achieves analytical sensitivity of 10 parasites per ml of blood for detection of *T. congolense* savannah type and *T. brucei*, and 100 parasites per ml of blood for detection of *T. vivax* in infected mouse blood. The ITS1 TD PCR was evaluated on cattle experimentally infected with *T. congolense* during an investigational new veterinary trypanocide drug efficacy study. ITS1 TD PCR demonstrated comparable performance to microscopy in verifying trypanocide treatment success, in which parasite DNA became undetectable in cured animals within two days post-treatment. ITS1 TD PCR detected parasite recrudescence three days earlier than microscopy and had a higher positivity rate than microscopy (84.85% versus 57.58%) in 66 specimens of relapsing animals collected after treatments. Therefore, ITS1 TD PCR provides a useful tool in assessment of drug efficacy against *T. congolense* infection in cattle. As the assay bears the potential for detection of mixed infections, it may be applicable for drug efficacy studies and diagnostic discrimination of *T. vivax* and *T. congolense* against other pathogenic trypanosomes, including *T. brucei*, *T. evansi* and *T. equiperdum*.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

\* Corresponding author. Tel.: +32 3 247 63 71; fax: +32 3 247 63 73.

E-mail address: [pbuscher@itg.be](mailto:pbuscher@itg.be) (P. Büscher).

## 1. Introduction

Animal African trypanosomoses (AAT) are infectious diseases of livestock that kill approximately 3 million cattle each year, with a further 50 million at risk of the disease (FAO, 2003). AAT contribute to poor meat and milk production, poor growth of young stock and reduction in fertility (Shaw et al., 2014). The causative agents of AAT are flagellated protozoa of the *Trypanosoma* genus. *Trypanosoma congolense*, *T. vivax* and *T. brucei* cause wasting disease nagana, mainly in ruminants, and are transmitted by tsetse flies in sub-Saharan Africa. *T. vivax* also occurs in Latin America where it is transmitted by other blood sucking flies. *T. evansi* causes surra in camels, horses and ruminants and occurs in Northern and Eastern Africa, Latin America, South East Asia and sporadically in Southern Europe. *T. equiperdum* causes dourine that is a sexually transmitted ubiquitous disease of equines (Hoare, 1972). *T. congolense* is considered the economically most important species that induces severe pathology in cattle, including anaemia, weakness and immune depression (Sharpe et al., 1982; Mwangi et al., 1990). Emergence of drug resistance presents a threat to the control of trypanosomosis and has triggered research on new compounds against African trypanosomes (Chitanga et al., 2011; Mungube et al., 2012).

The UK government's Department for International Development (DFID) has funded an AAT drug, diagnostics and vaccine discovery programme administered by the Global Alliance for Livestock Veterinary Medicines (GALVmed) a not-for-profit company based in Edinburgh, Scotland. A key effort in this programme is to discover and develop new trypanocide treatments to overcome current issues of toxicity and resistance which are inherent to the existing trypanocides (homidium, isometamidium, and diminazene) that have been used in Africa for more than 50 years. Given the significant resources and effort invested in human African trypanosomosis (HAT) drug discovery by groups such as the Drugs for Neglected Diseases initiative (DNDi) the opportunity exists to explore candidate trypanocidal compounds for efficacy against AAT. GALVmed has defined trypanocide compound progression criteria and Target Product Profile (TPP) criteria for AAT trypanocides to aid in their development and is progressing suitable candidates into development for therapeutic and prophylactic treatments of AAT (<http://www.galvmed.org/2012/04/trypanosomosis/>). Development includes assessing the efficacy of suitable candidate trypanocide compounds against drug-resistant *T. congolense* and *T. vivax* isolates in the target species, namely cattle.

Trypanocide efficacy studies determine parasite clearance in cattle following treatment. These studies are however hampered by the generally low analytical sensitivity of microscopical trypanosome detection methods resulting in a recommended 100 days of post treatment follow-up with frequent examination of the blood (Eisler et al., 2001). A commonly used microscopic test and considered "gold standard" is the haematocrit centrifugation technique (HCT, (Woo, 1970)) with a generally accepted detection limit of about 500 parasites per ml of blood. As HCT detects living trypanosomes, the test should be performed

quickly after specimen collection. To overcome the limitations of microscopical analysis, molecular methods have been introduced in compound efficacy studies against AAT. For example, a PCR targeting a *Trypanosomatidae*-specific 18S rDNA was able to detect *T. evansi* parasites with a median of 10 days earlier than HCT in goats that relapsed more than 100 days after treatment (Gillingwater et al., 2011). Another PCR, targeting multi-copy internal transcribed spacer 1 (ITS1) rDNA sequences, may serve the same purpose but with the advantage that it is able to identify the trypanosome species or subgenus by the amplicon length polymorphism (McLaughlin et al., 1996).

ITS1, situated between the conserved 5.8S and 18S genes encoding the ribosomal RNA subunits, occurs in approximately 100–200 copies per genome of a trypanosome. Due to variation in sizes of ITS1 amongst different *Trypanosoma* taxa, discrimination between species or subgenus is possible in a single run (McLaughlin et al., 1996; Desquesnes et al., 2001). ITS1 or nested ITS1/ITS2-based PCR assays have proven useful in trypanosomosis diagnosis and in epidemiological studies (Njiru et al., 2005; Cox et al., 2005; Thumbi et al., 2008; de Clare Bronsvort et al., 2010; Fikru et al., 2012). The authors claimed that the universal ITS-based PCR assays reduce cost and time of running several species-specific assays, especially in large-scale studies. ITS1 PCR that was used in an epidemiological survey in Ethiopia revealed a five-fold higher detection rate for *T. vivax* compared to HCT (Fikru et al., 2012). However, evaluation of the assay as a test of cure has not been reported and these ITS1 PCR assays are prone to non-specific amplification, particularly with bovine blood (unpublished observations). Therefore, the assay presented in this study was further refined for optimal performance. "Touchdown" PCR approach, which employs more stringent primer-template hybridisation temperatures, was introduced to enhance assay specificity. In Touchdown PCR, the annealing temperature during the first PCR cycles is well above the predicted optimal annealing temperature of the primers thus favouring the amplification of the specific target sequence. In the following PCR cycles, the annealing temperature is gradually lowered to more permissive temperatures. By maintaining the same high number of amplification cycles as in a classical PCR, the sensitivity is not compromised (Don et al., 1991; Korbie and Mattick, 2008).

The objectives of this study were (1) to develop an ITS1 "Touchdown" PCR for multi-taxon detection of the *Trypanosoma* genus and (2) to evaluate the performance of this ITS1 TD PCR as test of cure in an efficacy study designed to evaluate novel trypanocidal compounds in cattle infected with *T. congolense*.

## 2. Materials and methods

### 2.1. Ethical clearance

Animal studies at the Institute of Tropical Medicine (ITM, Antwerp, Belgium) received ethical clearance from the Veterinary Ethics Committee at ITM (BMW 2012-1 and BMW 2013-7).

Animal studies at ClinVet (Bloemfontein, South Africa) received ethical approval from the ClinVet Animal Ethics Committee (CAEC) authorising the research facility to conduct three studies CV12/884; CV 12/928; CV12/885. Animals were housed and cared for in accordance with national and international legislation, and local animal regulatory requirements.

## 2.2. Parasite strains

The following *T. congolense* strains were used for infection of cattle: KONT 2/133, KONT 2/151 (Mamoudou et al., 2008), Maputo 31], Maputo 02] (unpublished).

For test development, the following strains were used for infection of mice: *T. congolense* YOBO 2038, TRT 17, Lion 209 (Delespau et al., 2008), STIB 212 (Nantulya et al., 1980); *T. vivax* ILRAD 700, IL 1392 (Leefflang et al., 1976); *T. brucei brucei* AnTat 1.1, *T. b. gambiense* AnTat 9.1 (Van Meirvenne et al., 1975), *T. evansi* RoTat 1.2 (Bajyana Songa and Hamers, 1988), *T. b. rhodesiense* ETat 1.2 (Van Meirvenne et al., 1976), *T. equiperdum* OVI (Barrowman, 1976) and *T. theileri* Melsele (Verloo et al., 2000).

## 2.3. Trypanocide efficacy studies

The trypanocide efficacy studies were carried out at ClinVet, Bloemfontein, South Africa. All cattle were *Trypanosoma*-susceptible, castrated males and females of the Friesian–Holstein breed. The animals were at least four months of age and had been weaned for at least two months. Animals originated from a tsetse and *Trypanosoma*-free area, were negative for trypanosomosis (PCR-RFLP assay for *T. congolense* and *T. vivax*, (Geysen et al., 2003)) and negative for *T. theileri* on blood smear performed at ClinVet International (Pty) Ltd. Animals were identified by ear tags, were weighed at regular intervals throughout the study and were given a standard diet of hay and a commercial, supplemented, concentrate feed (without added antimicrobial agents) sufficient to support growth rates of approximately 700 g/day in healthy growing cattle. Animals were housed in a fully enclosed, purpose-built, fly-proof facility for cattle containing 36 flexible pens.

For this evaluation, blood samples from a total of 57 animals across 3 studies were used. Twelve animals were non-infected and 45 animals were infected with a single *T. congolense* strain per animal. Fresh heparinised blood (0.1 mL) from an infected donor animal containing the pathogen (infective dose of approximately 100,000 parasites as determined by counting using the Urigrass disposable counting chamber (Menarini Diagnostics, Austria)) was administered by slow intravenous injection into the jugular vein of recipient calves within 15 min after collection. For assessment of trypanocide efficacy in study CV12/885, two groups of six animals each, namely groups A and B (i.e. 12 out of the 45 infected cattle) were infected with the drug resistant strain KONT 2/133 and each group was given a different trypanocide 9 days after infection when obvious parasitaemia and anaemia were present together with variable clinical signs. Day of first treatment administration was designated day 0. Animals were then monitored for 100 days according to Eisler et al. (2001).

Animals in group B relapsed and were retreated with another trypanocide 19 days after the first treatment. Animals in Group A did not relapse after treatment.

From the infected animals, blood for PCR and parasite detection was collected on either 9 or 5 days pre-infection (45 trypanosome negative control specimens) and at 14 days post-infection and prior to trypanocide administration (45 trypanosome positive control specimens). From the non-infected animals, blood was taken at days corresponding with the sampling from the infected animals (24 trypanosome negative control specimens). These specimens were used for evaluation of the sensitivity and specificity of ITS1 TD PCR.

From groups A and B, blood samples for PCR and HCT were collected after treatment with trypanocides, on 1, 2, 3, 4, 6, 10, 16, 23, 30 and 44 days post-treatment. These specimens were used for evaluation as a “test of cure” of ITS1 TD PCR. Blood was drawn from the jugular vein into K<sub>2</sub>-EDTA vacutainer tubes. The blood was stored at –80 °C for subsequent DNA extraction. For parasite detection, blood was drawn into heparinised collection tubes, transferred to 6 heparin-containing capillary tubes and centrifuged for 6 min at 13,000 × g. The buffy coat was examined under a microscope for the presence of living trypanosomes according to Woo (1970).

For assessing the specificity of the PCR primers, non-infected blood collected on heparin or on Na<sub>2</sub>-EDTA from bovine, goat, dog, horse, human and mouse was used.

## 2.4. DNA purification

Total genomic DNA was extracted from 200 µl of blood using the High Pure PCR Template Purification Kit (Roche Applied Sciences) according to the manufacturer's instructions, except that bound DNA was eluted with 60 µl elution buffer instead of 200 µl. Purified DNA was stored at –80 °C. Each round of DNA extraction included a negative control (PCR-grade water) and a positive control (parasite DNA-spiked blood) alongside the bovine blood specimens. For determination of the analytical sensitivity, trypanosomes were grown in mice and parasites were counted in a Urigrass cell counting chamber. Since bovine blood was not readily available at the ITM in Antwerp, 10-fold serial dilutions of parasites were prepared in 1 ml volumes of ice-cold freshly collected naïve human or mouse blood. Two hundred µl of the thus prepared blood series were subjected to DNA purification as described above. For assessment of analytical specificity, trypanosomes were grown in mice. Trypanosomes were separated from the blood by anion exchange chromatography (Lanham and Godfrey, 1970) and subjected to DNA purification with the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions.

## 2.5. ITS1 TD PCR and agarose electrophoresis analysis

ITS1 primers for detection of the *Trypanosoma* genus were described in Claes et al. (2007). Primer sequences are: ITS1-Forward 5'-TGT AGG TGA ACC TGC AGC TGG ATC, ITS1-Reverse 5'-CCA AGT CAT CCA TCG CGA CAC GTT. PCR assays were performed in a Biometra T3000 cyler

(Germany). Each reaction contained a final volume of 50  $\mu$ l, including 5  $\mu$ l of template DNA, 200  $\mu$ M of each dNTP (Eurogentec), 0.2  $\mu$ M of each primer (Biolegio), 1 unit of Hot Star Taq Plus DNA polymerase (Qiagen), 1  $\times$  Coral Load PCR buffer, and 0.1 mg/ml acetylated bovine serum albumin (Promega). PCR amplification was carried out over 40 cycles at the following conditions: initial activation step at 95  $^{\circ}$ C for 5 min; three amplification cycles with 94  $^{\circ}$ C for 30 s, 70  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; three cycles with 94  $^{\circ}$ C for 30 s, 69  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; three cycles with 94  $^{\circ}$ C for 30 s, 68  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; three cycles with 94  $^{\circ}$ C for 30 s, 67  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; three cycles with 94  $^{\circ}$ C for 30 s, 66  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; three cycles with 94  $^{\circ}$ C for 30 s, 65  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s; 22 cycles with 94  $^{\circ}$ C for 30 s, 64  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s. A final extension step occurred at 72  $^{\circ}$ C for 5 min. Each PCR run included a positive control (0.5 ng parasite DNA) and a no-template negative control (5  $\mu$ l PCR-grade water).

PCR products were resolved by electrophoresis at 6 V/cm in 2% (w/v) agarose gels stained with 0.5  $\mu$ g/ml ethidium bromide, and photographed under ultraviolet light.

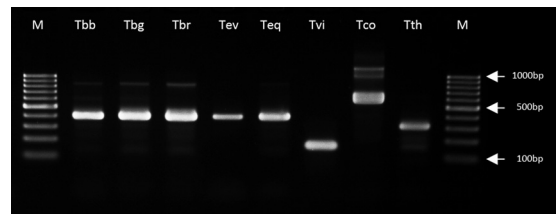
## 2.6. Vertebrate cytochrome b PCR

On all blood specimens that were negative in the ITS1 TD PCR, a PCR for vertebrate cytochrome b was performed (Kocher et al., 1989; Fikru et al., 2012). A positive result with vertebrate cytochrome b PCR indicates that a negative ITS1 TD PCR result of the same specimen is not due to poor DNA quality or presence of inhibitors.

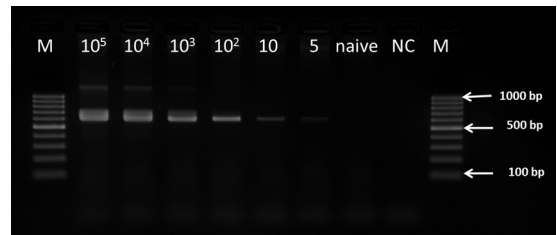
## 3. Results

### 3.1. Analytical specificity and sensitivity of ITS1 TD PCR

The ITS1 TD PCR assay conditions were optimised in order to obtain maximal specificity and sensitivity using parasite-infected mouse blood, and non-infected blood from mouse, human, bovine, goat, horse and dog. No cross-reactivity was observed with the non-infected blood specimens, while the ITS1 TD PCR allowed detection and differentiation of the trypanosome taxa by amplicon length polymorphism. Indeed, the assay generated amplicons of the expected sizes: 612 bp with *T. congolense* Savannah type, 165 bp with *T. vivax* and 391–393 bp with the *Trypanozoon* subgenus, including *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi*, and *T. equiperdum*. The non-pathogenic *T. theileri* could be discriminated by a PCR amplicon of approximately 300 bp (Fig. 1). To assess the lower detection limit according to the trypanosome taxon, 10-fold serial dilutions of live parasites in 1 ml aliquots of naïve human blood were used. At 200  $\mu$ l blood per sample, ITS1 TD PCR achieved an analytical sensitivity of 10 parasites/ml blood or 0.2 parasite equivalent/reaction with *T. congolense* (Fig. 2), *T. brucei* and *T. evansi* (data not shown). The analytical sensitivity of ITS1 TD PCR for *T. vivax* was 100 parasites/ml blood or 2 parasite equivalent/reaction (Fig. 3).



**Fig. 1.** Ethidium bromide-stained agarose gel electrophoresis of PCR products generated from genomic DNA of *T. b. brucei* (Tbb), *T. b. gambiense* (Tbg), *T. b. rhodesiense* (Tbr), *T. evansi* (Tev), *T. equiperdum* (Teq), *T. vivax* (Tvi), *T. congolense* Savannah type (Tco) and *T. theileri* (Tth). M: 100 bp incremental markers.

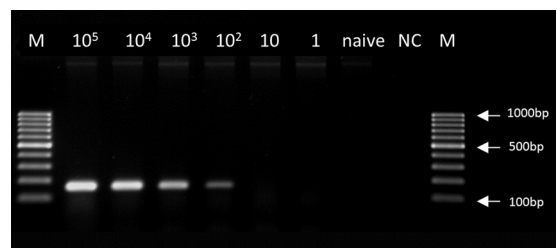


**Fig. 2.** Ethidium bromide-stained agarose gel electrophoresis of ITS1 TD PCR products of *T. congolense* Savannah type genomic DNA extracted from blood.  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , 10, 5:  $10^5$  parasites per ml blood,  $10^4$  parasites per ml blood,  $10^3$  parasites per ml blood,  $10^2$  parasites per ml blood, 10 parasites per ml blood and 5 parasite per ml blood, respectively; naïve: non-infected blood; NC: no-template negative control. M: 100-bp incremental markers. Each reaction contained the equivalent of 20  $\mu$ l of blood.

### 3.2. Performance of ITS1 TD PCR on clinical specimens

A total of 246 blood specimens from 57 cattle were examined with ITS1 TD PCR and HCT and results were interpreted with reference to their infection status with *T. congolense* Savannah or Kilifi type. Specimens that were negative in ITS1 TD PCR were verified with the vertebrate cytochrome b PCR and were all positive (data not shown).

Firstly, specificity and sensitivity of ITS1 TD PCR were evaluated on a collection of 114 reference specimens (69 non-infected and 45 infected specimens) from 57 cattle of known disease status. Specificity of ITS1 TD PCR was 100%, which was in agreement with that of HCT. Sensitivity of ITS1 TD PCR at 14 days post infection was 100%, and that of HCT was 97.8%.



**Fig. 3.** Ethidium bromide-stained agarose gel electrophoresis of ITS1 TD PCR products of *T. vivax* gDNA extracted from blood.  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , 10, 1:  $10^5$  parasites per ml blood,  $10^4$  parasites per ml blood,  $10^3$  parasites per ml blood,  $10^2$  parasites per ml blood, 10 parasites per ml blood and 1 parasite per ml blood, respectively; naïve: non-infected blood; NC: no-template negative control. M: 100 bp incremental markers. Each reaction contained the equivalent of 20  $\mu$ l of blood.

**Table 1**

HCT and ITS1 TD PCR results obtained post treatment in the cattle of group A. The animals were treated on day 0. – denotes negative test result, + denotes positive test result.

Days post treatment	Animal identification												
	CVB 197		CVB 192		CVB 190		CVB 214		CVB 193		CVB 191		
	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR	
1	–	–	–	–	<b>+</b>	–	–	–	–	–	<b>+</b>	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	–	–	–	–
16	–	–	–	–	–	–	–	–	–	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	–	–	–

Discordant results between HCT and ITS1 TD PCR are indicated in bold.

Secondly, ITS1 TD PCR was evaluated as a test of cure on the collection of 132 post-treatment specimens in 12 cattle (groups A and B) that were treated and followed up. Four out of six animals in group A became negative within 1 day post treatment with both ITS1 TD PCR and HCT (Table 1). After 1 day of treatment, one animal was positive in ITS1 TD PCR and negative in HCT, and another animal was positive in HCT and negative in ITS1 TD PCR. From 2 days post-treatment till the end of the sampling period (44 days after first treatment), all animals were negative in both the ITS1 TD PCR and the HCT. In group B, all six animals were occasionally positive in ITS1 TD PCR and/or HCT during the follow-up period between 1 and 16 days after treatment and even after retreatment (on day 19) up to day 44 after treatment (Table 2). The parasite detection rate of ITS1 TD PCR after two treatments in animal group B was higher than that of HCT. Positivity rate of ITS1 TD PCR was 84.85% (56/66), while HCT was 57.58% (38/66). The ITS1 TD PCR could detect relapse up to three days earlier than microscopical parasite detection.

#### 4. Discussion

Livestock production is considered the main lifeline for millions of families in many sub-Saharan countries.

**Table 2**

HCT and ITS1 TD PCR results obtained post treatment in the cattle of group B. The animals were treated on day 0 and rescue treatment with a second trypanocide was given at day 19 after the first treatment. – denotes negative test result, + denotes positive test result.

Day post treatment	Animal identification											
	CVB 198		CVB 200		CVB 199		CVB 204		CVB 205		CVB 202	
	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR	HCT	PCR
1	–	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	–	–	–	<b>+</b>	–	<b>+</b>
2	–	–	<b>+</b>	<b>+</b>	–	<b>+</b>	<b>+</b>	–	–	<b>+</b>	–	–
3	–	–	–	<b>+</b>	–	<b>+</b>	–	–	–	<b>+</b>	–	<b>+</b>
4	–	<b>+</b>	<b>+</b>	<b>+</b>	–	<b>+</b>	–	<b>+</b>	–	–	–	<b>+</b>
5	–	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	–	<b>+</b>	<b>+</b>	<b>+</b>	–	<b>+</b>
6	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	–	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
10	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
16	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
23	–	–	–	–	–	–	–	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
30	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	–	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>
44	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>+</b>

Discordant results between HCT and ITS1 TD PCR are indicated in bold.

However, the emergence of drug resistant trypanosomes presents a serious threat to agriculture in the regions. Therefore, studies on drug resistance and development of novel compounds against trypanosomes are necessary for effective control. These studies greatly benefit from rapid and cost-efficient molecular tools to detect the presence of trypanosomes. Compared to microscopy, PCR-based assays have the advantage that they are more amenable to high throughput processing and that specimens can be stored longer term. In addition, differentiation between trypanosome taxa by microscopy is much more cumbersome than with molecular methods, such as PCR. This study presents an improved ITS1-based PCR assay for diagnosis of trypanosomosis and for efficacy assessment of trypanocidal compounds where the detection of genomic rDNA of *Trypanosoma* specific ITS1 serves as surrogate for parasite detection but with higher analytical sensitivity. The same primer sequences were employed in a previous survey on AAT in Ethiopia (Fikru et al., 2012). However, the reaction mixture and cycling conditions of the ITS1 TD PCR are refined for optimal sensitivity and specificity. The “Touchdown” approach that employs more stringent primer-template hybridisation conditions is introduced to minimise potential non-specific amplifications. It favours amplification of desirable products during early cycles that



will out-compete potential non-specific products during the remaining cycles (Korbie and Mattick, 2008).

For *T. congolense*, the newly developed ITS1 TD PCR has a lower detection limit of 10 parasites/ml blood while for *T. vivax*, this is 100 parasites/ml blood. A similar difference in detection limits between *T. congolense* and *T. vivax* has been observed in other PCRs such as the original ITS1 PCR and a real-time PCR targeting the 18S rDNA sequence (unpublished results) and might be due to a lower copy number of target sequences in the *T. vivax* genome compared to *T. congolense*. Currently, the 18S based PCR-RFLP is the FAO recommended test for detection and differentiation of multiple trypanosome taxa (Geysen et al., 2003). The 18S PCR-RFLP requires one or two amplification steps and a restriction digestion step. On the other hand, the ITS1 TD PCR needs a single amplification step and achieves a comparable detection limit, is thus less-time consuming and more cost-effective for detection of *T. congolense*, *T. vivax* and the three species within the *Trypanozoon* subgenus *T. brucei*, *T. evansi* and *T. equiperdum*.

The data obtained with the blood specimens of experimentally infected cattle demonstrated that ITS1 TD PCR had comparable performance to microscopy in revealing the absence or presence of a *T. congolense* infection prior to treatment. After curative treatment, the ITS1 TD PCR became negative in all animals within no more than two days, thus proving the rapid clearance of DNA from dead parasites in the blood. The discordant result at day 1 post-treatment in animal CVB 190, with positive HCT and negative ITS1 TD PCR was confirmed with a negative result in the 18S-based real-time PCR developed in our laboratory (unpublished), but cannot be explained. In the animals that relapsed after treatment, ITS1 TD PCR achieved a higher positivity rate and was able to detect recrudescence of parasitaemia several days earlier than HCT. Therefore, ITS1 TD PCR can be considered more reliable than microscopy in assessment of trypanocide efficacy and bears the potential to allow shortening of the post-treatment follow-up that is currently recommended to be 100 days.

In summary, the ITS1 TD PCR offers an alternative tool for trypanosome diagnosis and assessment of compound efficacy against *T. congolense* in cattle. The ability to detect and differentiate multiple *Trypanosoma* taxa in a single run, with the potential to identify mixed infections, indicates the application of the ITS1 TD PCR for research on other pathogenic trypanosomes, such as *T. vivax*, *T. evansi*, *T. equiperdum* and *T. brucei*.

#### Conflict of interest statement

None of the authors has any financial or personal relationship that could inappropriately influence or bias the content of the paper.

#### Acknowledgements

This research was supported by the Global Alliance for Livestock Veterinary Medicines (GALVmed) with funding from the UK Government's Department for International Development (DFID) as part of GALVmed's Animal African Trypanosomosis Program (DFID Programme: Controlling

African Animal Trypanosomosis (AAT) (Aries code 202040-101). The authors are grateful to Dr. Filip Claes for the *in silico* work. We thank Fatima Balharbi, Nicolas Bebronne and Stijn Rogé for technical assistance. For the veterinary clinical trials (animal husbandry, veterinary supervision, project management and data management), we acknowledge the ClinVet International (Pty) Ltd staff. *T. congolense* isolates Maputo 31J, Maputo 02J were kindly provided by Luis Neves (Centre for Biotechnology, University of Eduardo Mondlane, Maputo, Mozambique).

#### References

- Bajjana Songa, E., Hamers, R., 1988. A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. *Ann. Soc. Belg. Med. Trop.* 68, 233–240.
- Barrowman, P.R., 1976. Observations in the transmission, immunology, clinical signs and chemotherapy of dourine (*Trypanosoma equiperdum* infection) in horses, with special reference to cerebrospinal fluid. *J. Vet. Res.* 43, 55–66.
- Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J., Delespau, V., 2011. High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. *PLoS Negl. Trop. Dis.* 5, e1454.
- Claes, F., Agbo, E.C., Radwanska, M., te Pas, M.F.W., Büscher, P., 2007. Molecular markers for the different (sub)-species of the *Trypanozoon* subgenus. In: Crowther, J.R. (Ed.), *Developing Methodologies for the use of Polymerase Chain Reaction in the Diagnosis and Monitoring of Trypanosomiasis*. International Atomic Energy Agency, Vienna, pp. 217–233.
- Cox, A., Tilley, A., McOdimba, F., Fyfe, J., Eisler, M., Hide, G., Welburn, S., 2005. A PCR based assay for detection and differentiation of African trypanosome species in blood. *Exp. Parasitol.* 111, 24–29.
- de Clare Bronsvort, B.M., von Wissmann, B., Fèvre, E.M., Handel, I.G., Picozzi, K., Welburn, S.C., 2010. No gold standard estimation of the sensitivity and specificity of two molecular diagnostic protocols for *Trypanosoma brucei* spp. in Western Kenya. *PLoS ONE* 5, e8628.
- Delespau, V., Dinka, H., Masumu, J., Van den Bossche, P., Geerts, S., 2008. Five-fold increase in *Trypanosoma congolense* isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia. *Drug Resist. Updates* 11, 205–209.
- Desquesnes, M., McLaughlin, G., Zoungrana, A., Davila, A.M., 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *Int. J. Parasitol.* 31, 610–614.
- Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., Mattick, J.S., 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* 19, 4008.
- Eisler, M.C., Brandt, J., Bauer, B., Clausen, P.-H., Delespau, V., Holmes, P.H., Ilemobade, A., Machila, N., Mbwambo, H., McDermott, J., Mehltz, D., Murilla, G., Ndung'u, J.M., Peregrine, A.S., Sidibé, I., Sinyangwe, L., Geerts, S., 2001. Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. *Vet. Parasitol.* 97, 171–182.
- FAO, 2003. In: Kamuanga, M. (Ed.), *Socio-Economic and Cultural Factors in the Research and Control of Trypanosomiasis*. FAO/WHO/IAEA/OAU-IBAR, Rome, pp. 1–67.
- Fikru, R., Goddeeris, B.M., Delespau, V., Moti, Y., Tadesse, A., Bekana, M., Claes, F., De Deken, R., Büscher, P., 2012. Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse- as well as non-tsetse-infested regions of Ethiopia: a reason for concern? *Vet. Parasitol.* 190, 355–361.
- Geysen, D., Delespau, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. *Vet. Parasitol.* 110, 171–180.
- Gillingwater, K., Gutierrez, C., Bridges, A., Wu, H., Deborggraeve, S., Ekangu, R.A., Kumar, A., Ismail, M., Boykin, D., Brun, R., 2011. Efficacy study of novel diamidine compounds in a *Trypanosoma evansi* goat model. *PLoS ONE* 6, e20836.
- Hoare, C.A., 1972. *The Trypanosomes of Mammals*. Blackwell Scientific Publications, Oxford, pp. 749.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. U. S. A.* 86, 6296–6300.
- Korbie, D.J., Mattick, J.S., 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat. Prot.* 3, 1452–1456.

- Lanham, S.M., Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp. Parasitol.* 28, 521–534.
- Leeflang, P., Buys, J., Blotkamp, C., 1976. Studies on *Trypanosoma vivax*: infectivity and serial maintenance of natural bovine isolates in mice. *J. Parasitol.* 6, 413–417.
- Mamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S., 2008. Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. *Acta Trop.* 106, 115–118.
- McLaughlin, G.L., Ssenyonga, S.S., Nanteza, E., Rubaire-Akiki, W.O., Hansen, R.D., Vodkin, M.H., Novak, R.J., Gordon, V.R., Montenegro-James, S., James, M., Avile, H., Armijos, R., Santrich, C., Weigle, K., Saravia, N., Wozniak, E., Gaye, O., Mdachi, R., Shapiro, S.Z., Chang, K.-P., Kakoma, I., 1996. PCR-based detection and typing of parasites. In: Azcel, M.A., Alkan, M.Z. (Eds.), *Parasitology for the 21 Century*. CAB International, Wallingford Oxon, pp. 261–287.
- Mungube, E.O., Vitouley, H.S., Allegye-Cudjoe, E., Diall, O., Boucoum, Z., Diarra, B., Sanogo, Y., Randolph, T., Bauer, B., Zessin, K.H., Clausen, P.H., 2012. Detection of multiple drug-resistant *Trypanosoma congolense* populations in village cattle of south-east Mali. *Parasites Vectors* 5, 155.
- Mwangi, D.M., Munyua, W.K., Nyaga, P.N., 1990. Immunosuppression in caprine trypanosomiasis: effects of acute *Trypanosoma congolense* infection on antibody response to anthrax spore vaccine. *Trop. Anim. Health Prod.* 22, 95–100.
- Nantulya, V.M., Doyle, J.J., Jenni, L., 1980. Studies on *Trypanosoma (nannomonas) congolense*. III. Antigenic variation in three cyclically transmitted stocks. *Parasitology* 80, 123–131.
- Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C., Davila, A.M., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitol. Res.* 95, 186–192.
- Sharpe, R.T., Langley, A.M., Mowat, G.N., MacAskill, J.A., Holmes, P.H., 1982. Immunosuppression in bovine trypanosomiasis: response of cattle infected with *Trypanosoma congolense* to foot-and-mouth disease vaccination and subsequent live virus challenge. *Res. Vet. Sci.* 32, 289–293.
- Shaw, A.P., Cecchi, G., Wint, G.R., Mattioli, R.C., Robinson, T.P., 2014. Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomiasis in Eastern Africa. *Prev. Vet. Med.* 113, 197–210.
- Thumbi, S.M., McOdimba, F.A., Mosi, R.O., Jung'a, J.O., 2008. Comparative evaluation of three PCR base diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. *Parasites Vectors* 1, 46.
- Van Meirvenne, N., Janssens, P.G., Magnus, E., 1975. Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei*. I. Rationalization of the experimental approach. *Ann. Soc. Belg. Med. Trop.* 55, 1–23.
- Van Meirvenne, N., Magnus, E., Janssens, P.G., 1976. The effect of normal human serum on trypanosomes of distinct antigenic type (ETat 1 to 12) isolated from a strain of *Trypanosoma brucei rhodesiense*. *Ann. Soc. Belg. Med. Trop.* 56, 55–63.
- Verloo, D., Brandt, J., Van Meirvenne, N., Büscher, P., 2000. Comparative in vitro isolation of *Trypanosoma theileri* from cattle in Belgium. *Vet. Parasitol.* 89, 129–132.
- Woo, P.T.K., 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. *Acta Trop.* 27, 384–386.