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Specific detection and identification of African trypanosomes in bovine peripheral blood by means of a PCR-ELISA assay

Leyda Cabrera [a,b], Jacob De Witte [b], Björn Victor [b], Lieve Vermeiren [b], Mirko Zimic [c], Jef Brandt [b], Dirk Geysen [b,*]

[a] Alexander von Humboldt Institute of Tropical Medicine (IMTAvH), Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, San Martín de Porras, Lima 31, Peru
[b] Prince Leopold Institute of Tropical Medicine (ITM), Nationalestraat 155, Antwerp B-2000, Belgium
[c] Laboratories of Research and Development (LIDS), Faculty of Science, Universidad Peruana Cayetano Heredia, Av. Honorio Delgado 430, San Martín de Porras, Lima 31, Peru

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ABSTRACT

The aim of the present study was to develop a PCR-ELISA assay for the detection and differentiation of the main African pathogen trypanosomal species present in peripheral blood of cattle. The proposed methodology allows to specifically differentiate *Trypanosoma congolense*, *Trypanosoma vivax* and the subgenus *Trypanozoon*, by means of a sensitive universal PCR amplifying trypanosome DNA followed by an ELISA-based hybridization with three highly specific probes. The semi-nested PCR had a sensitivity of 15 fg, 15 fg, and 0.15 fg of DNA from *T. vivax*, *T. congolense*, and *Trypanosoma brucei brucei*, respectively that is sufficient to detect parasites in blood during the chronic phase of the disease. Biotinylated second round asymmetric PCR amplification products were used in an ELISA set up using three species-specific probes for the diagnosis of *T. congolense* (type Riverine, Kilifi or Savannah), *T. vivax* and *T. brucei brucei*. A factor O.D. of 0.082 was determined on blood samples from bovines ($n = 18$) from a non-endemic area in Africa. In a pilot study of blood samples of naturally and experimentally *Trypanosoma* infected cattle previously characterized by PCR-RFLP ($n = 42$), a high rate of concordance (93.3%) was found between PCR-RFLP and PCR-ELISA. There is a good ratio between positive and negative O.D. values (3.00 vs. 0.1) and the technique can also be used to distinguish mixed infections.

1. Introduction

Field studies on trypanosome prevalence require sensitive and reliable methods for detection and differentiation of parasites in both the mammal host and the tsetse vector. Molecular based *Trypanosoma* diagnosis have used several amplification targets and most PCR assays are specific for only one species or subgenus group and often have not been validated on field samples (reviewed in Desquesnes and Dávila, 2002). Satellite DNA in nuclear minichromosomes has been successfully used in *Trypanozoon* (Moser et al., 1989; Masiga et al., 1992), *Trypanosoma vivax* (Masiga et al., 1992), *Trypanosoma evansi* (Artama et al., 1992), *Trypanosoma simiae*, and *Trypanosoma congolense* types Savannah, Forest and Kilifi (Masiga et al., 1992). Nuclear mini-exon genes also present as multicopy genes have been a preferred target for *Trypanosoma cruzi* and *T. vivax* (Ventura et al., 2001) diagnostic assays. Other repetitive sequences have been investigated, for example in *T. vivax* (Masake et al., 1997; Clausen et al., 1998; Morlais et al., 2001) whereas kinetoplast minicircles DNA have been a good target in the development of specific primers for *T. evansi* detection (Masiga and Gibson, 1990; Artama et al., 1992; Diall, 1993).

In the veterinary field, however, DNA diagnosis with these specific primers requires as many PCRs as *Trypanosoma* species (or types) could be potentially present in the

* Corresponding author. Tel.: +32 3 2476 264; fax: +32 3 2161 431.
E-mail address: dgeysen@itg.be (D. Geysen).

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host, so that often up to five PCR reactions are required on blood samples from cattle.

An important simplification in the species-specific characterization of trypanosomes was the replacement of several PCR assays by a single one that allowed detection of "pan-trypanosomes" (Desquesnes et al., 2001). This assay used the ITS 1 region of the multicopy ribosomal genes as target but lacked sensitivity in *T. vivax* detection. Later, Geysen et al. (2003) developed a PCR-RFLP to amplify part of the 18S small sub-unit ribosomal gene (Ssu-rDNA). This gene is a mosaic with regions of high and low homology and is present as a multicopy locus in tandem arrays. These characteristics make this gene an ideal target for use in diagnosis and characterization of "pan-trypanosomas" by PCR-RFLP. However, PCR-RFLP is not a convenient technique to be used in field studies as it is a relatively complex and time-consuming procedure.

On the other hand, Masake et al. (2002) used a PCR-ELISA format for the detection of *Trypanosoma brucei* and *T. vivax*, but not for *T. congolense*. This approach was based on separated PCR reactions for each species, which is costly in time and reagents. However, an important advantage of the format is the presence of ELISA expertise and equipment in most national and provincial veterinary laboratories in developing countries.

The objective of the present study was to develop a simple PCR-ELISA assay for the specific detection and identification of pathogenic African trypanosomes in bovines. A semi-nested PCR detecting all trypanosome DNA extracted from bovine peripheral blood samples was followed by an ELISA hybridization using three specific probes to detect *T. congolense* (types Riverine, Kilifi and Savannah), *T. vivax* and subgenus *Trypanozoon* (*T. brucei* spp., *T. evansi* and *Trypanosoma equiperdum*).

2. Materials and methods

2.1. Blood samples

Bovine blood sample collection and storage were done either as blood spots on filter paper from buffy coat (BC) or whole blood (WB), or as such in EDTA tubes. Group 1 blood samples ($n = 42$) were obtained from cattle experimentally infected with *Trypanosoma* (experimental animals, ITM, Belgium) or found positive on the PCR-RFLP assay after natural exposure in Ivory Coast Group 2 blood samples ($n = 18$) were obtained from negative bovines from a non-endemic zone in the Southern Province of Zambia. All these samples had already been characterized and diagnosed as positive or negative by PCR-RFLP using the method developed by Geysen et al. (2003).

Bovine blood samples spiked with trypanosomes were used in the standardization of the PCR-ELISA assay. For this purpose, blood obtained from trypanosome infected mice was serially diluted adding non-infected bovine blood to obtain a range of 1/10 to 1/100.000 trypanosomes/ml (for details see Geysen et al., 2003) and each blood dilution was spotted as BC or WB on filter paper and stored until DNA extraction.

2.2. DNA extraction

A classical phenol–chloroform extraction followed by ethanol precipitation was used on the trypanosome spiked blood samples and on most of bovine blood samples. Other protocols for DNA extraction included QIAamp kit (Qiagen, US) and saponine–chelex extraction, modified from Plowe as described by Devos and Geysen (2004).

2.3. Design of specific probes and primer 18 ST FF1

Five sequences of the 18S small sub-unit ribosomal gene were used in the probes and primer design (GeneBank accession numbers *T. congolense* type Riverine: U22319; *T. congolense* type Kilifi: U22317; *T. congolense* type Savannah: AJ009146; *T. brucei brucei*: M12676; and *T. vivax*: U22316).

CLUSTAL W 1.82 (Chenna et al., 2003) was used to align the five fragments (approximate length of 700 bp). Probes with similar characteristics (location around the same gene region, 20-nt length, adequate Tm and not hybridizing intra- or inter-specifically with amplification products *in silico*) were selected with PRIMER PREMIER v5 program (Biosoft International, CA, USA), followed by BLAST analysis to confirm the specificity.

The design of a new primer 18ST FF1 (abbreviated FF1) was necessary to avoid hybridization of the *T. congolense* probe with a region downstream of primer nF2. This forward primer FF1 replaced nF2 in first and second round amplifications of the nested PCR.

2.4. PCR-ELISA

2.4.1. Primers and probes

The reverse primers nR2 (gtg tct tgt tct cac tga cat tgt agt g) and nR3 (tgc gcg acc aat aat tgc aat ac) were used as described in Geysen et al. (2003). A new forward primer FF1 (cag att gag tgt tct ttc tcg atc c), and probes: *T. congolense* (ggc ggc gct atc aca cgg gg), *T. brucei* (ccc aac ggt ggt cgt cat cc) and *T. vivax* (gcc caa cgc cgt ccg cat cc) were developed and synthesized by Eurogentec (Belgium). Biotinylated primers were used in the ELISA procedure.

2.4.2. Semi-nested PCR

An identical protocol was used as for the former PCR, except for a 3 mM Mg concentration and annealing temperature of 51 °C for the primerpair FF1/nR3 in the first round (40 cycles of synthesis). The second round is an asymmetric PCR using double quantity of the biotinylated primer nR2 of the primerpair FF1/nR2 at 54 °C annealing temperature and 1.65 mM Mg concentration (25 cycles of synthesis).

2.4.3. Direct PCR (FF1/nR2-bio, 40 cycles)

Five microliters of DNA extract was amplified in a reaction mix prepared in the same way as for the second PCR round. The cycling and temperatures of amplification were also the same as those used in the second round, except that 40 cycles were used instead of 25.

2.4.4. ELISA protocol

The ELISA hybridization format was based on Orle and Weiss (1998). The DNA probes were diluted in a fresh 1 M ammonium acetate solution (stock sterilized with a 0.22 μm filter) using the following optimised probe concentrations: 12.5 ng/100 μl for *T. congolense* and *T. brucei* probes and 100 ng/100 μl for *T. vivax*. The probes were separately coated onto a 96-well ELISA plate (NUNC #269787) placing 100 μl of diluted probe into each well and incubating overnight at 37 °C. Thereafter, the plate was washed twice using a 1:10 dilution of washing buffer (buffer Cobas 10×, Roche), the last wash solution was removed and the plate left drying out at room temperature for 2–5 h and stored at 4 °C in sealed bags until used.

The biotinylated PCR products (FF1/nR2-bio) were denatured by heating at 95 °C for 5 min. Hybridization was done in the pre-coated plate using 100 μl of denaturation/hybridization buffer (5× SSPE, 0.1% SDS, 30% formamide) and 15 μl of denatured amplicon per well followed by an incubation at 37 °C during 1 h. The plate was washed five times with diluted 1:10 buffer (Cobas) and dried, whereafter 100 μl/well Horseradish Peroxidase enzyme conjugated to Streptavidin (Vector Laboratories, CA, USA) with a concentration of 1 μg/ml was added. A new round of five times washing of the plate with Cobas and drying was followed by the addition of 100 μl/well TMB prepared according to the manufacturer's instructions (TMB peroxidase substrate kit, Vector Laboratories) and incubated for 10 min in the dark (giving a blue coloration to the positive wells). The reaction was stopped with 2N sulphuric acid and the optical density (O.D.) of the resulting yellow color was measured in an ELISA reader (Ascent, Thermo LabSystems, Multiskan EX) at 450 nm.

The ELISA analysis of bovine blood samples was done in duplicate for each probe, including PCR negative controls (water). Positive controls consisted of DNA extracts from pure *T. congolense*, *T. brucei brucei*, and *T. vivax* infections in mice.

3. Results

3.1. Standardization of nested PCR with primer 18 ST FF1

Good amplification with the new primer FF1 in both PCR rounds was obtained using DNA from the three species of interest *T. congolense*, *T. vivax*, and *T. brucei* and also *T. evansi*, a species belonging to subgenus *Trypanozoon*. No amplification products were found using host bovine DNA. The expected lengths for first (data not shown) and second round (Fig. 1) amplifications were confirmed experimentally. For the second round PCR with the primerpair FF1/nR3 the expected lengths of amplification products are as follows: 330 bp for *T. congolense* types Savannah and Riverine, 344 bp for *T. congolense* type Kilifi, 320 bp for *T. brucei*, and 305 bp for *T. vivax*.

3.1.1. Specificity of PCR amplifications

BLAST analysis of primer FF1 predicted that this primer would also anneal to the 18S gene region of other trypanosomatide species including *T. rangeli* and *T. cruzi* as well as other kinetoplastid species of the family *Bodonidae*.

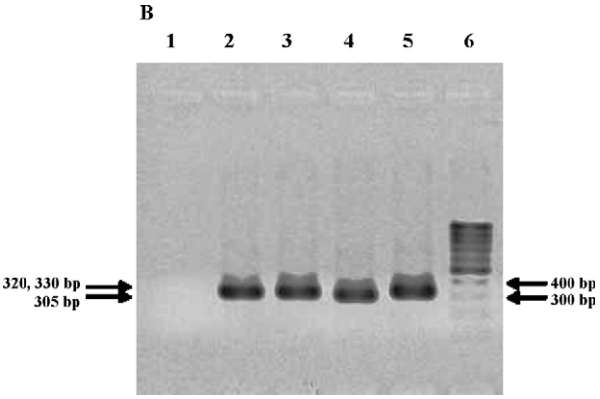

Fig. 1. Nested PCR amplification results with new forward primer 18 ST FF1. The 2% agarose gel shows second round FF1/nR2 amplification products for the following species: lane 2, *T. brucei brucei*; lane 3, *T. evansi*; lane 4, *T. vivax*; and lane 5, *T. congolense* type Savannah. Lane 1 contains a negative control (reaction mix without DNA) and lane 5 contains MW marker of 100 bp.

The FF1 primer pairs were tested for their specificity using 10–50 ng/μl DNA of several hemoparasites both of bovine and human origin. Non-specific amplification products were obtained with species like *Trypanosoma theileri*, *T. simiae*, *T. cruzi* and *Leishmania braziliensis*. Other species of hemoparasites like *Babesia rodhaini*, *Theileria parva* and *Eperythrozoon* gave also some aspecific amplification products whereas *Babesia divergens* and *Ehrlichia* were negative (figure not shown).

The sensitivity of the new 18S primer FF1 was compared with the PCR-RFLP described by Geysen et al. (2003). PCRs were run using *T. vivax*, *T. congolense*, and *T. brucei brucei* serial dilutions starting with a concentration of 0.3 ng/ml of trypanosomal DNA.

Using the nested PCR format with the new FF1 set of primers, sensitivities were two orders of magnitude better than found with the PCR-RFLP method for *T. congolense* and *T. brucei*: 15 fg, and 0.15 fg, respectively. For *T. vivax*, the sensitivity was the same (15 fg) for both the new and the PCR-RFLP set of primers. The repeatability, estimated in a kappa test, was 71% for three repetitions on 93 observations (with proportions of positives ranging between 47 and 51%).

3.2. ELISA optimisation

3.2.1. Curve of probe concentration in ELISA

The optimal initial probe concentration for plate coating was determined using twofold concentration range from 6.5 ng to 200 ng per 100 μl of ammonium acetate solution using 100 μl of dilution per well. The probe concentration that yielded the greatest O.D./parasite ratio or DNA dilution was determined using a combination of both parameters (probe concentration vs. trypanosomes or DNA dilutions). We used a direct PCR, employing second round primers FF1/nR2-bio, with 40 amplification cycles (see PCR's Section, for experimental details) in order to test the system with a less concentrated amplification product.

For *T. congolense*, a clear decrease of the substrate signal (O.D.) with increasing probe concentration was observed

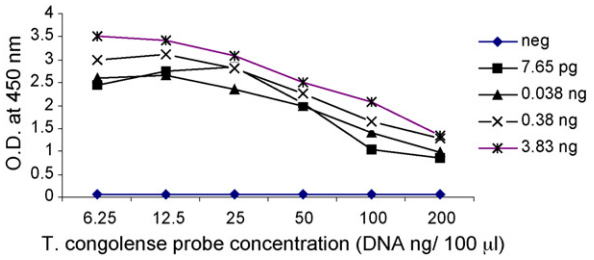

Fig. 2. Graph of calibration curves for initial *T. congolense* probe concentration for plate coating using direct PCR (FF1/nR2 bio, 40 cycles) amplification products of parasite DNA serial dilutions. A negative control for the amplification reaction was also included.

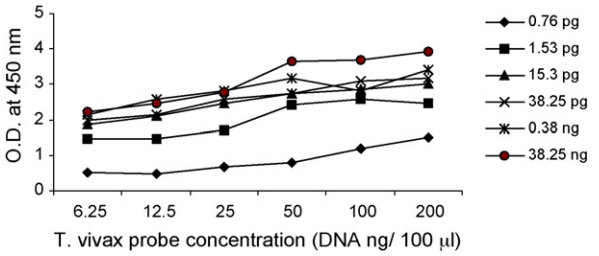

Fig. 4. Graph of calibration curves for initial *T. vivax* probe concentration for plate coating using direct PCR (FF1/nR2 bio, 40 cycles). Amplification products were prepared with parasite DNA serial dilutions as shown in the legend to the right of the graph.

(Fig. 2) and this result was seen for every parasitic DNA concentration. A concentration of 12.5 ng/100 µl was chosen for coating of plates with the *T. congolense* probe. For the *T. brucei* probe (Fig. 3) the O.D. signal was almost constant with increasing concentrations of the probe, and a 12.5 ng/100 µl concentration was chosen for plate coating with the *T. brucei* probe.

Finally, in a similar way, the *T. vivax* probe concentration of 100 ng/100 µl was chosen as optimal concentration for the *T. vivax* probe (results shown in Fig. 4).

In general, as these experiments for optimal probe concentration were performed using amplicons from a direct PCR, relatively lower O.D. readings were obtained than those from amplicons of a nested PCR, as shown in Figs. 4 and 5. It can be seen that the O.D. increases with increasing concentrations of amplified DNA in the direct PCR (Fig. 6). However, when a nested PCR was used, O.D. values almost overlap regardless of the DNA concentration and the system always works to saturation (Fig. 5). For example, the O.D. signals for the lower amounts of amplified DNA (0.76 pg and 1.53 pg) improved when using a nested PCR format in comparison with a direct format. An important consequence is that low concentrations of DNA are favoured by using a nested PCR format.

3.2.2. Analytical specificity of probes in ELISA

Once optimal concentrations for each probe were established, specificity assays for each of the three designed probes were carried out. DNA from several trypanosomatides as well as other parasites was used: *T. theileri*, *T. simiae*, *T. cruzi*, *L. braziliensis*, *B. rodhaini*, *B. divergens*, *T. parva*, *Ehrlichia* sp. and *Eperythrozoon*. For example, using the *T. brucei* probe, ELISA was done in duplicate with PCR products using DNA from the above species and also from *T. congolense* and *T. vivax*. The corresponding O.D. readings ranged from 3.43 to 4.03 for the *T. brucei* and *T. evansi* group and from 0.047 to 0.067 for the rest of non-Trypanozoon group DNA extracts.

Consequently, despite the fact that several species yielded a positive although aspecific signal in PCR, O.D. readings were negative in the ELISA assay with the *T. brucei* probe except for *T. evansi* derived amplicons belonging to the same subgenus *Trypanozoon* that gave a positive signal as expected.

Likewise, comparable results as for the *T. brucei* probe were found with the *T. congolense* and *T. vivax* probes, i.e. the probes only hybridized with amplicons from the corresponding homologous species (O.D. approx. 3), but not with those from the other parasites (O.D. <0.1). The experiments confirmed the high specificity of the three probes.

3.2.3. Analytical sensitivity of the PCR-ELISA assay

To evaluate the sensitivity of the PCR-ELISA assay, nested PCR's amplification products (FF1/R2-biotine) obtained from reactions with serial dilutions of *T. vivax*, *T. congolense*, and *T. brucei brucei* DNA starting with a concentration of 0.3 ng/µl were exposed to an ELISA plate coated with the three specific probes.

The sensitivity obtained was 15 fg, 15 fg, and 0.15 fg of *T. vivax*, *T. congolense* and *T. brucei* DNA used in the amplifications, respectively; i.e. the same sensitivity corresponding to the PCR amplifications (see Section 3.1).

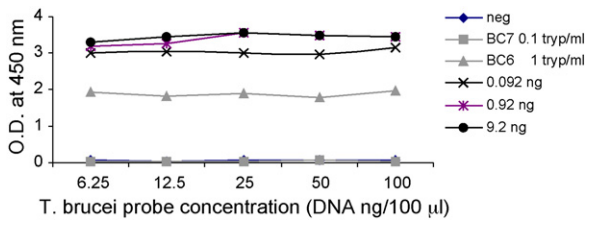

Fig. 3. Graph of calibration curves for initial *T. brucei* probe concentration for plate coating using direct PCR (FF1/nR2 bio, 40 cycles) amplification products of: BC7 and BC6, 0.1 and 1 trypanosome/ml of blood and of: 0.092, 0.92 and 9.2 ng of parasite DNA. A negative control for the amplification reaction was also included.

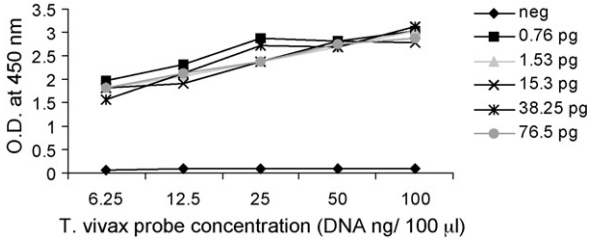

Fig. 5. Graph of calibration curves for initial *T. vivax* probe concentration for plate coating using nested PCR (first and second round with sets FF1/nR3 and FF1/nR2-bio, respectively). Amplification products were prepared with parasite DNA serial dilutions as shown in the legend to the right of the graph. A negative control for PCR reaction is also shown.

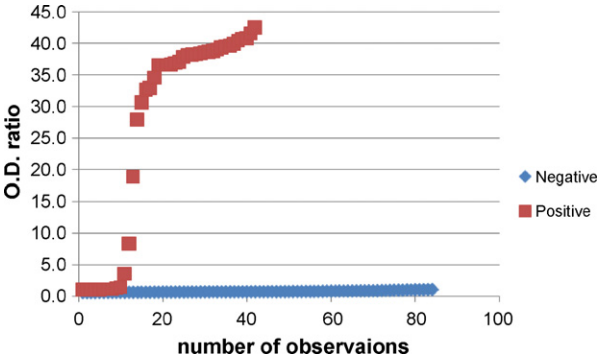

Fig. 6. Optical density (O.D.) ratio distribution in Group 1 of animals ($n = 42$, 126 observations) infected with *T. congolense*, *T. brucei* and *T. vivax* and tested by three-probe ELISA-PCR assay. The ratios were obtained dividing the O.D. for each sample by factor 0.082 (see Section 3.2.4 for details). A sample was considered positive when ratio O.D. was greater than 1.0, otherwise was negative.

3.2.4. Normalization of O.D.

An O.D. factor value was determined by evaluating PCR-ELISA results using negative samples from cattle ($n = 18$) from a non-endemic zone in Africa (Southern Province of Zambia). These samples had been previously analyzed by PCR-RFLP, and found negative for the three *Trypanosoma* species (Geysen D., personal communication). For this purpose, the average of O.D.s from these negative samples tested with the three probes plus 3 SD was calculated (0.082).

The above factor was then used to determine the O.D. ratio for samples of Group 1 and Group 2. A sample was considered positive when O.D. ratio was greater than 1.0, otherwise was negative.

3.3. Evaluation of PCR-ELISA using bovine infected samples

Bovine samples from Group 1 and Group 2 were used to evaluate the developed assay. Group 1 ($n = 42$) contained blood sample DNA from trypanosome infected bovines, and Group 2 ($n = 18$) consisted of blood DNA samples of bovines from a non-endemic zone in Africa (Zambia). The PCR-ELISA assay showed a good performance for the three species *T. congolense*, *T. brucei* and *T. vivax* (Fig. 6). Most marginally positive O.D. ratios with values of 1.1 corresponded to the samples in which mixed infections were detected as discussed in the following section.

3.4. Concordance between PCR-ELISA and PCR-RFLP performances

Using data from the trypanosome infected and negative groups ($n = 60$), an analysis of concordance was carried out in order to compare the performance of PCR-ELISA vs. PCR-RFLP. Concordance analysis demonstrated that the results obtained with both methods are strongly associated, with a concordance of 93.3%. The difference between both assays was due to a better diagnostic sensitivity by the PCR-ELISA test to detect mixed infections. 10 out of 42 samples in Group 1 showed mixed infections: 6 samples were *T. congolense* and *T. vivax* positive (3 samples with medium or strong O.D. signals for both species, 3 samples with marginally positive signals for *T. vivax*); 3 samples were *T. congolense* and *T. brucei* positive (with marginally positive signals for both species); and only 1 sample was *T. brucei* and *T. vivax*, in which *T. vivax* signal was marginally positive.

The repeatability of the PCR-ELISA, estimated in a kappa test, was 98% for 2 repetitions on 93 observations, of which 91 and 89 were negative in the first and the second test, respectively.

4. Discussion

The objective of the present study was to develop a specific molecular technique to identify and differentiate the three pathogenic African trypanosomes present in blood of infected bovines. The development of an approach based on PCR-ELISA would be better suited for large-scale epidemiological studies.

In the present study, the ELISA format was used for the detection of a hybridization signal using a PCR targeting the 18S ribosomal gene and further analysis with trypanosome-specific capture probes. The 18S gene is part of the transcriptional unit of small sub-unit ribosomal genes (SSu-rDNA) and is present in approximately 100–200 copies. The gene's mosaic structure is ideal for designing universal primers in the highly conserved regions and species-specific probes in the polymorphic regions.

The 18S gene has already been used as target for molecular characterization of African pathogenic trypanosomes in bovines (Geysen et al., 2003), but using a PCR-RFLP approach. The new PCR-ELISA assay has been based on the same 18S area but the forward primer had to be adapted. The new primer pairs were universal and efficiently amplified DNA of *T. congolense*, *T. brucei brucei* and *T. vivax* among other species. On the other hand, the assay also efficiently amplified in both first and second rounds the DNA from the bovine parasites *T. theileri* and *T. simiae* and human pathogens as *T. cruzi* and *L. braziliensis*.

The analytical sensitivity of the modified (FF1) nested PCR was up two orders of magnitude greater than that found with the former nested PCR. The sensitivity comparison in the modified nested PCR showed that *T. brucei* is better amplified than *T. vivax* and *T. congolense*. The improved sensitivity of this nested PCR increased the sensitivity of the PCR-ELISA and is in the range of fg of target DNA.

The ELISA format consisted in the colorimetric detection of the amplification product of the second round (FF1/nR2) according to a protocol of Orle and Weiss (1998). An optimization of this protocol consisted in using one instead of two biotinylated primers, as described in the PCR-ELISA study of Masake et al. (2002). This increased the O.D. signal. One explanation for this increase could be the possibility of re-association between the positive and negative strands of the amplicons is greater when both strands have been evenly biotinylated, than when only one strand is biotinylated (probably due to the small difference in the mol. wt of biotin being 514 Da). In this way some of

these positive strands would be lost after the washing step, decreasing the likelihood to hybridize with the probe attached to the plate. Further improvement was obtained by using an asymmetric nested PCR approach producing the biotinylated positive strand in excess.

In the optimization of probe concentrations for plate coating, the behavior of each probe was found different. The *T. congolense* probe showed a decreasing signal of optical density with increasing probe concentration, *T. brucei* showed a constant signal while *T. vivax* showed an increasing signal. In the case of *T. congolense*, this behavior could be explained by a tendency of this probe to form homodimers ($\Delta G = -9.9$ k-cal/mol), whereas for *T. brucei* and *T. vivax* the explanation would be the presence of different stages in the saturation curve.

The three probes were found to be highly specific for each of the three main species of interest: *T. congolense*, *T. brucei* and *T. vivax* with the absence of cross-reactions. In the case of *T. congolense* the same probe recognizes the most important types within this species, i.e. Riverine/warf, Kilifi and Savannah. On the other hand, a complete homology of the amplified region exists between *T. brucei* spp., *T. evansi* and *T. equiperdum*, i.e. species within the subgenus *Trypanozoon*, preventing a differential identification. However, considering that *T. evansi* and *T. brucei brucei* species within this *Trypanozoon* subgenus only infect cattle and that these species have mainly a separate geographic occurrence in Africa, this homology is of less practical importance.

The potential of differentiating the three main African trypanosome species is particularly important in veterinary epidemiology as there is the possibility of simultaneously finding more than the three *Trypanosoma* species in a single bovine animal. However, the high specificity of the probes excludes hybridization with other amplification products from pathogenic or non-pathogenic (*T. theileri*) trypanosomatides. This is an important point, as it was demonstrated that primer 18 ST FF1 could also amplify the 18S region of most if not all trypanosomatides, and other kinetoplastids in general (e.g. *Bodo* sp. in contaminated tap-water). This test feature is considered an asset as it will allow the amplification and identification of new species, especially when analyzing extracts obtained from the fly vector. On the other hand, it was found that when assay was applied to field samples it could easily detect mixed infections. The most common and strong combined infection was that of *T. congolense* and *T. vivax*. The combined infections *T. congolense* plus *T. brucei* or *T. brucei* plus *T. vivax* were also present but less frequently and with weaker signals, but show the advantage of PCR-ELISA to detect very early infections and on the other hand show its better performance in comparison of PCR-RFLP assay that detected only *T. congolense* plus *T. vivax* mixed infections giving a two total mixed infections against a total of 10 mixed infections detected by PCR-ELISA.

A logistic advantage of using the ELISA format lies in the fact that most provincial and national laboratories in Africa are already equipped with ELISA readers. An additional practical feature of this ELISA assay is the use of a non-labeled probe coated onto the plate by ammonium acetate solution. Whereas in an alternative protocol by Masake et al. (2002), it is the labeled amplicon which is attached to the plate, so that each PCR amplicon must be coated onto the plate and hybridized sequentially to specific probes, making that approach more demanding in time and cost.

An evaluation of the PCR-ELISA assay using samples from cattle infected with trypanosomes, showed a clear difference between values of animals diagnosed as trypanosome positive and those diagnosed as negative, making use of an ELISA reader almost not necessary. The technique worked well with samples using different DNA extraction protocols and allows a clear identification of mixed infections, for example of *T. congolense* and *T. vivax*, which are reported of occurring naturally in field studies. The comparison of performance between PCR-ELISA vs. PCR-RFLP on bovine samples using concordance analysis showed that the ELISA format offers an equally good alternative for the PCR-RFLP.

As the main interest lies in the correct and fast diagnosis of the three pathogenic species of trypanosomes in cattle, this test offers a good combination of simplicity, accuracy, sensitivity and speed. The protocol is being evaluated on field samples from an epidemiological region in Africa. The technique could also be applied for detection of trypanosomes in the vector (*Glossina* spp.), and other mammal hosts such as camelides, equines, ovines, porcines and even in humans with sleeping sickness disease.

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