

The detection and PCR-based characterization of the parasites causing trypanosomiasis in water-buffalo herds in Venezuela

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Received 21 September 2004, Revised 15 February 2005,

Accepted 17 February 2005

The usefulness of PCR-based assays for detecting trypanosomiasis in water buffaloes and other livestock was explored, under field conditions, in Venezuela. The sensitivity and specificity of the assays, which were based on established primer pairs (21-mer/22-mer and ILO1264/ILO1265), were evaluated, partly by comparison with the results of parasitological tests (stained bloodsmears and microhaematocrit centrifugation) and immunological assays (IFAT) run in parallel. The optimised PCR-based assays showed a sensitivity of 10 pg DNA. The use of the 21-mer/22-mer primer pair gave a test that was specific for species in the subgenus *Trypanozoon* (including *Trypanosoma evansi*), whereas use of ILO1264/ILO1265 produced a test that was specific for *T. vivax*. The results of a hybridization assay using *T. evansi*-DNA and *T. vivax*-DNA probes indicated no cross-hybridization between the *T. evansi* and *T. vivax* PCR products.

The results of the bloodsmear examinations, microhaematocrit centrifugations (MHC) and IFAT indicated that 23 (6.7%), 39 (11.4%) and 135 (39.5%) of the 342 blood samples investigated (including 316 from water buffaloes) contained trypanosomes, respectively. The results of the PCR-based assays indicated that 68 (19.9%) of the same blood samples contained *T. vivax* (or at least *T. vivax* DNA), and that none contained *T. evansi* or any other member of the subgenus *Trypanozoon*. For the detection of trypanosomes, the assay therefore appeared almost twice as sensitive as the MHC. These results are the first on the molecular characterization of the trypanosomes infecting water buffaloes in Venezuela.

When the results of the MHC (which is the most practical, and frequently used, alternative detection method) were used as the gold standard, the PCR-based assay for *T. vivax* was found to have 100% sensitivity, 90.4% specificity, a positive predictive value of 0.57, a positive likelihood ratio of 10.45, and a negative likelihood ratio of 0.00. The

assay therefore appears a reasonable choice for detecting *T. vivax* in the mammalian livestock of Venezuela and elsewhere.

Trypanosoma (Duttonella) vivax and *Trypanosoma (Trypanozoon) evansi* are haemoprotozoan parasites that cause trypanosomiasis in livestock, and are among the most important constraints to animal production in Africa, Asia and Central and South America. Although *T. vivax* is the more important, causing considerable

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disease among cattle and other ungulates, *T. evansi* is the more wide-spread, thanks to the ubiquity of susceptible hosts (Zhang and Baltz, 1994). In Venezuela, both species are only transmitted mechanically, by tabanids and other biting flies such as *Stomoxys calcitrans* and *Haematobia irritans* (Rivera, 1996).

There have only been a few reports of trypanosomiasis in water buffaloes (*Bubalus bubalis*) in Venezuela, the first being that of Toro *et al.* (1978), who detected trypanosomes in buffaloes from the Orinoco Delta, using parasitological and serological methods, without identifying the parasite to species level. Water buffaloes, which originate in Asia, have only been bred as livestock in Venezuela for a few decades, and little is known about the prevalence and geographical distribution of trypanosomiasis among them, or the trypanosome species involved. Bovine trypanosomiasis is characterized by a variable clinical spectrum, ranging in severity from subclinical, asymptomatic infection to a chronic wasting disease, with weight loss, anaemia, reproductive disturbances, and death. As mass treatment is generally too expensive to be practical, reliable diagnostic tests are essential if trypanosome infections in cattle and buffaloes are to be treated before significant morbidity can occur (Obasi *et al.*, 1999).

Traditionally, current trypanosome infections are detected by parasitological methods such as the examination of wet or dry and stained bloodsmears or microhaematocrit centrifugation (MHC) followed by the examination of the buffy coat. Unfortunately, these methods show poor sensitivity, and low parasitaemias, such as those that frequently occur in chronically infected animals or early in an infection, can easily be missed. Such methods also require microscopists who are well trained in morphological differentiation, if the parasites detected are to be identified to species. Serological methods based on antibody detection can be more sensitive than microscopy but cannot be used to differentiate current from past

infections, and most cannot be used to distinguish between the various species of trypanosome that might be present. Moreover, several factors associated with the host's immune response must be carefully considered (Nantulya, 1990; McManus and Bowles, 1996).

Recently, molecular biology has become increasingly important in diagnosis, and several assays based on the detection of parasite-specific DNA sequences have already been developed for the trypanosomiasis. With the use of the appropriate primers, PCR-based assays can be used not only to detect low parasitaemias in livestock and reservoir hosts but also to detect infection in the vectors and to identify the species of trypanosome present (Wuyts *et al.*, 1995; Masake *et al.*, 1997). The main aim of the present study was to evaluate a PCR-based diagnostic method, under field conditions in Venezuela, by using it, in parallel with bloodsmear examination, MHC and an IFAT, to detect and identify the trypanosomes in water buffaloes and other livestock. The PCR-based assays used were also standardized, under laboratory conditions, for the detection of *T. evansi* and *T. vivax*.

MATERIALS AND METHODS

PCR Standardization for the Detection of *Trypanosoma evansi* and *T. vivax* parasite DNA

DNA of each of the following trypanosome stocks, which were kindly donated by Dr F. Bringaud of the Laboratory of Molecular Parasitology, University of Bordeaux, France, was used as template: *T. brucei brucei* (stock 345); *T. brucei rhodesiense* (stock 362); *T. brucei gambiense* type 1 (stock Zakaria); *T. brucei gambiense* type 2 (stock TH2-78E); *T. evansi* (stock Kenya); *T. equiperdum* (stock BoTat); *T. congolense* (stock IL3000); and *T. vivax* (stock Y481). In addition, DNA from reference strains of *T. vivax* (ILO2160) and *T. evansi* (TE0) — which were the generous gifts of Drs A. M.

Davila (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) and H. Perez (Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela), respectively — was also studied.

PCR-based assays

To test the sensitivity of the PCR-based assays, preliminary tests, with various concentrations of the primers, were conducted using decreasing concentrations of purified DNA from *T. evansi* TE0 or *T. vivax* ILO2160, as appropriate. The specificity of each of the assays was explored by running the PCR with DNA from each of these two reference strains and the other trypanosome stocks available, and by a cross-hybridization analysis. Each PCR-based amplification was performed in a 25- μ l reaction mixture containing 22 μ l PCR Supermix [22 mM Tris-HCl (pH 8.4), 55 mM KCl; 1.65 mM MgCl₂, 0.22 mM of each of the four 2'-deoxynucleoside 5'-triphosphates, and 22 U *Taq* DNA recombinant polymerase/ml; Gibco-BRL, Gaithersburg, MD], 1 μ l of each of the two primers, and 1 μ l DNA template. Each reaction mixture was overlaid with 25 μ l mineral oil (Sigma) before amplification was carried out in a PTC-100[®] thermocycler (MJ Research, Waltham, MA). The thermocycler was set to give a pre-incubation at 95°C for 5 min, followed by 40 cycles, each of 1 min at 94°C, 1.5 min at 55°C and then 1 min at 72°C, followed by a final extension at 72°C for 5 min.

Primers

The four oligonucleotides used as primers had been described previously. The '21-mer' (5'-TGC AGA CGA CCT GAC GCT ACT -3') and '22-mer' (5'-CTC CTA GAA GCT TCG GTG TCCT-3') primers were described by Wuyts *et al.* (1995), for use in a PCR-based assay, with DNA tandem sequence as target, that yields a product of approximately 227 bp. The other primer pair used, ILO1264 (5'-CAG CTC GGC GAA GGC CAC-3') and ILO1265 (5'-TCG CTA

CCA CAG TCG CAA TC-3'), was described by Masake *et al.* (1997) for use in an assay — based on the complimentary DNA sequence (apparently present in all stocks of *T. vivax*) coding for the antigen used in a diagnostic ELISA — that yields a product of approximately 400 bp.

Electrophoresis and hybridization conditions

A 10- μ l sample of the products from each assay was subjected to electrophoresis (60 min at 75 V) on 1.5% agarose in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, at pH 8.3). The gels were stained with ethidium bromide (0.5 μ g/ml) and the bands visualized by ultra-violet trans-illumination.

For the hybridization analyses, the amplification products resolved by agarose-gel electrophoresis were denatured, transferred bidirectionally (Smith and Summer, 1980) onto a nylon Hybond[™] N⁺ transfer membrane (Amersham Biosciences, Little Chalfont, U.K.) and hybridized with the appropriate ³²P-labelled probes [produced by random primer labelling using the Megaprimer DNA labelling kit (Amersham Biosciences)]. Both the *T. evansi* TE0 PCR-product (Te-DNA) and the *T. vivax* ILO2160 PCR-product (Tv-DNA) were used as probes, under conditions of medium stringency [with 2 \times sodium-citrate-sodium-chloride buffer (SSC), 2% SDS, 2 \times Denhardt's solution, and 100 μ g salmon sperm/ml, at 67°C], as previously described (Kukla *et al.*, 1987). After hybridization, the filters were washed at medium stringency, in 2 \times SSC/2% SDS, for 1 h at 67°C, with at least three changes of buffer, and then examined by autoradiography, with an intensifying screen, at -80°C.

Blood Samples

Blood samples were collected from 342 domestic animals in Venezuela: 316 water buffaloes from four farms in Guarico state, and 15 cattle, six horses and five sheep from a farm in Apure state. The livestock sampled

were selected at random from those present on the farms. Two samples were obtained from the jugular vein of each animal, using the Venoject® system (Terumo Medical, Somerset, NJ): one sample without anticoagulant and another to which sodium citrate (0.129 M) was added as anticoagulant. All the samples were kept at 4°C and protected from direct sunlight. Within 6 h of its collection, each citrate-treated blood sample was checked for trypanosomes by parasitological methods (see below) and genomic DNA was extracted from the sample, for testing in the PCR-based assay. Sera were separated from the coagulated blood samples, within 12 h of the sample collection, and stored frozen at -20°C until they could be tested for evidence of trypanosome infection, in IFAT (see below).

Diagnosis of Trypanosome Infection by Parasitological Techniques

Wet and dry bloodsmears

A 10- μ l aliquot of each citrate-treated blood sample was placed on a microscope slide, covered with a coverslip and checked for trypanosomes under a light microscope, at $\times 250$. In addition, thin smears were prepared, each with 5 μ l blood, and dried, fixed in methanol, stained with Giemsa and checked for trypanosomes at a magnification of $\times 1000$ (Parra and Vizcaino, 1976).

Microhaematocrit centrifugation

All citrate-treated blood samples were assessed by MHC. Briefly, microhaematocrit capillary tubes were three-quarters filled with blood, sealed and centrifuged at 12,000 $\times g$ for 5 min. The region of each centrifuged sample corresponding to the buffy coat was examined, while in the capillary tube, at low magnification ($\times 100$) for 2 min (Woo, 1969). As a further check, the capillary tubes containing the centrifuged samples were cut with a diamond pen so that each buffy coat could be extruded onto a microscope slide, covered with a coverslip and examined, as a wet

smear, for 5 min, using a dark-field/phase-contrast microscope at $\times 100$ (Murray *et al.*, 1977).

IFAT

Each serum sample was thawed at room temperature before a 10- μ l aliquot was run in an IFAT for detecting anti-trypanosomal antibodies.

Antigen

Fixed bloodstream trypomastigotes of *T. evansi* were used as the antigen in the IFAT. The trypomastigotes were collected from 10 CD1 mice, that had each been inoculated intraperitoneally with 10^3 trypanosomes. As soon as the examination of daily tail-blood samples revealed that the parasitaemias in the mice were peaking, each mouse was exsanguinated by cardiac puncture while under ether anaesthesia. The infected blood was collected in tubes containing sodium citrate (0.129 M) and then placed on the top of a DEAE-cellulose column so that the trypanosomes could be separated from the blood cells (Lanham and Godfrey, 1970) and then fixed with a mixture of cold acetone and formalin in saline (Katende *et al.*, 1987).

All the mice were checked for trypanosome infection before use (and found negative), kept in an insect-proof room, and given water and nutritional concentrate *ad libitum*. The experiments with the mice were approved by the Animal Care and Use Committee of the Universidad Central de Venezuela's School of Veterinary Science, and followed the U.S. National Institutes of Health's guidelines for the use of experimental animals.

Test procedure

Cross-reactivity means that the IFAT employed, although based on bloodstream trypomastigotes of *T. evansi*, gives positive reactions with antibodies against *T. evansi* or *T. vivax* (Voller *et al.*, 1975; Luckins, 1977). The conjugate used depended on the species providing the test sample. Rabbit

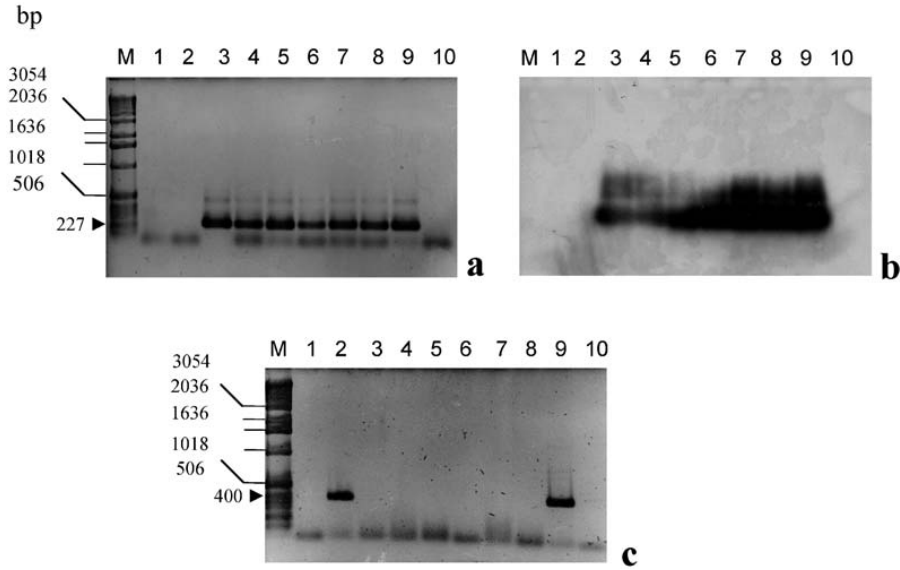


FIG. 1. The results of exploring the specificity of the primer sets and using DNA probes to check for cross-hybridization. In each PCR, 10 ng total genomic DNA from one, previously identified strain of *Trypanosoma* were used as template, with either the '21-mer/22-mer' primer pair (a) or the ILO1264/ILO1265 primer pair (c), each primer being used at a concentration of 0.23 pmol/ μ l. The PCR products were resolved on a 1.5%-agarose gel stained with ethidium bromide. The gel produced with the 21-mer/22-mer primer pair (a) was subjected to Southern blotting so that the filter could be hybridized with (32 P)-labelled probe based on *T. evansi* DNA (b). The DNA used as the templates came from *T. (Nannomonas) congolense* IL3000 (lane 1), *T. (Duttonella) vivax* Y481 (lane 2), *T. (Trypanozoon) brucei brucei* 345 (lane 3), *T. (Trypanozoon) brucei rhodesiense* 362 (lane 4), *T. (Trypanozoon) brucei gambiense* type 1 Zakaria (lane 5), *T. (Trypanozoon) brucei gambiense* type 2 TH2-78E (lane 6), *T. (Trypanozoon) evansi* Kenya (lane 7), *T. (Trypanozoon) equiperdum* BoTat (lane 8), or a positive control (lane 9) — *T. evansi* TE0 in (a) and *T. vivax* ILO2160 in (c). Lane 10, containing the PCR products when there was no DNA in the reaction mixture, represented the negative control. A 1-kb ladder of molecular-weight markers was also run in each gel (M)

anti-equine-IgG, rabbit anti-bovine-IgG and rabbit anti-sheep-IgG (whole molecule) were employed, as appropriate, each conjugated to fluorescein isothiocyanate. Sera (obtained from the *Universidad Central de Venezuela's* School of Veterinary Science) previously collected from animals known to be infected with *T. evansi* or *T. vivax* or to be uninfected were used as controls. The IFAT followed the standard methodology (García, 1988). The test sera that gave a titre of at least 1:80 were considered positive.

Use of the PCR-based Assays to Detect Animal Trypanosomiasis

After the PCR-based assays had been optimised, for both sensitivity and specificity, using the reference stocks of trypanosomes,

they were used to test the samples of citrated blood from the Venezuelan livestock.

Genomic DNA was isolated from each blood sample as described by Desquesnes and Tresse (1996). Briefly, after an initial centrifugation at slow speed (430 \times g for 10 min), 500–1000 μ l of the supernatant suspension were transferred to a sterile Eppendorf tube and centrifuged at high speed (12,000 \times g for 15 min). Each pellet so formed was mixed with 1 ml DNAzol® (Gibco–BRL). After gentle pipetting of the suspension, to lyse any cells, DNA was precipitated by the addition of 0.5 ml ethanol. Each sample was then mixed, by inversion, stored at room temperature for 3 min, and then centrifuged (4000 \times g for 1 min) at room temperature to

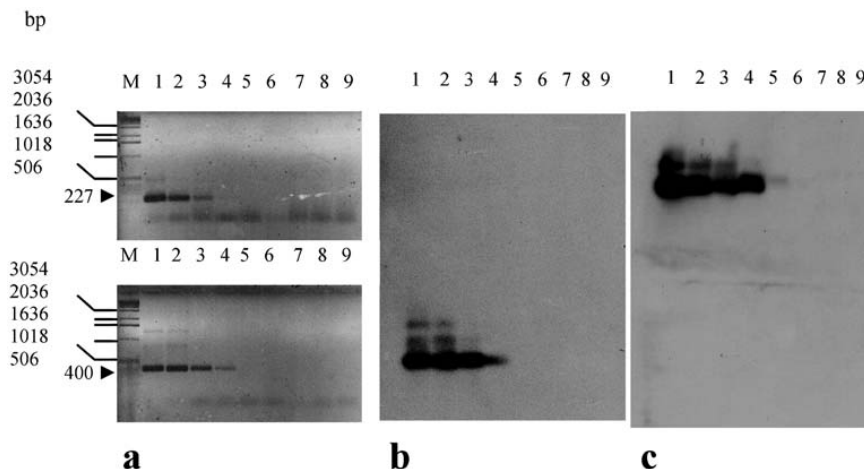


FIG. 2. The results of exploring the sensitivity of the 21-mer/22-mer and ILO1264/ILO1265 primer sets, with each primer at 0.23 pmol/ μ l. The PCR products generated from varying quantities of the genomic DNA of *T. evansi* TE0 and the 21-mer/22-mer primer pair (top), or of the genomic DNA of *T. vivax* ILO2160 and the ILO1264/ILO1265 primer pair (bottom) are shown in (a). The amount of DNA used as template was 10 ng (lane 1), 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1 pg (lane 5), 100 fg (lane 6), 10 fg (lane 7), 1 fg (lane 8) or none (lane 9). The PCR products were separated by electrophoresis on 1.5%-agarose gel stained with ethidium bromide. A 1-kb ladder of molecular-weight markers was also run in each gel (M). When the gels shown in (a) were hybridized, under conditions of medium stringency, with a (32 P)-labelled *T. vivax* probe (b) or a (32 P)-labelled *T. evansi* probe (c), the probes hybridized strongly to the homologous DNA but no cross-hybridization was observed.

pellet the DNA. The DNA precipitate was washed twice with 0.8–1.0 ml 95% ethanol, allowed to dry for 15 min at room temperature, and then solubilized in 8 mM NaOH. Two 1- μ l aliquots were then used as templates in the PCR-based assays, one with the 21-mer/22-mer primer pair and one with ILO1264/ILO1265, under the optimal conditions established in the preliminary trials.

Analysis of the Results

The performance of the PCR-based assay for *T. vivax* was determined using the results from the MHC (the most frequently used and often the most practical method to detect active trypanosome infections in livestock) as the 'gold standard'. The assay's sensitivity, specificity, positive predictive value (PPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) were determined (Thrusfield, 1990; Otero *et al.*, 2001).

RESULTS

Primer-pair Specificity in the PCR

The specificities of the 21-mer/22-mer [Fig. 1(a)] or ILO1264/ILO1265 [Fig. 1(c)] primer pairs were evaluated using reaction mixtures that each contained 0.23 pmol of each primer/ μ l, and 10 ng purified DNA from one of the identified strains of trypanosome. In reactions with the 21-mer/22-mer pair, DNA samples from all tested trypanosomes from the *Trypanozoon* subgenus yielded the expected amplicon of 227 bp, whereas there were no PCR products when DNA from *T. congolense* or *T. vivax* was used [Fig. 1(a)].

Use of the ILO1264/ILO1265 primer pair gave the expected amplicon of about 400 bp when the DNA template came from *T. vivax* Y481 or *T. vivax* ILO2160 but no product when the DNA came from trypanosomes in the *Nannomonas* or *Trypanozoon* subgenera. [When DNA from *T. congolense* was amplified using primers specific for this

species, the expected amplicon was generated (data not shown).]

Sensitivity of Detection and Optimal Concentration of Primers in the PCR

The minimal concentrations of DNA from *T. evansi* TE0 or *T. vivax* ILO2160 that were detectable in the PCR-based assays (with each primer at 0.23, 0.45 , 2.27 or 4.55 pmol/μl) were determined by running the assays with decreasing concentrations of the DNA. Although just 10 fg of purified DNA could be detected by using each primer at 4.55 pmol/μl, use of the two higher concentrations of primers produced artifacts (primer multimers) on the gels. Use of the lower primer concentrations (0.23 and 0.45 pmol/μl) produced no such artifacts, however, and still gave reasonable detection limits. With 0.23 pmol of each primer/μl, for example, the detection limit for assays based either on the 21-mer/22-mer primer pair and *T. evansi* DNA or the ILO1264/ILO1265 primer pair and *T. vivax* DNA was about 10 pg [Fig. 2(a)]. For all subsequent tests, therefore, each primer was used at 0.23 pmol/μl.

The level of homology between the amplicons generated using DNA from *T. evansi* TE0 and *T. vivax* ILO2160 was demonstrated by Southern-blot hybridization, with the Te-DNA and Tv-DNA as probes. The Tv-DNA probe specifically hybridized to the homologous *T. vivax* amplicons and showed no cross-hybridization with the *T. evansi*

amplicons, confirming the sequence specificity of the probe [Fig. 2(b)]. The Te-DNA probe [Figures 1(b) and 2(c)] recognized the amplicons generated from the genomic DNA of *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* types 1 and 2, *T. evansi* Kenya, *T. equiperdum* and *T. evansi* TE0 (i.e. all the members of the subgenus *Trypanozoon* that were investigated) but not those generated from the genomic DNA of *T. vivax*.

With the 21-mer/22-mer primers in the PCR-based assays each used at 0.23 pmol/μl and the quantity of the DNA template set at 10 pg, the results of the Southern blotting confirmed that the faint bands considered to represent low concentrations of the expected amplicon (indicating a DNA-detection limit of about 10 pg) were exactly that [Fig. 2(c)].

Serological, Parasitological and Molecular Evaluations of the Blood Samples

Having chosen a good primer concentration (0.23 pmol of each primer/μl) and established the specificities of the primer pairs, the PCR-based assays were used to test the 342 blood samples collected from Venezuelan livestock (Table 1). Use of the ILO1264/ILO1265 primer pair indicated that 68 (19.9%) of the samples were positive for *T. vivax*. Use of the 21-mer/22-mer primer pair indicated, however, that none of the samples was positive for *T. evansi* or any other member of the subgenus *Trypanozoon*.

TABLE 1. The results of the examination of the 342 blood samples collected from Venezuelan livestock, by the PCR-based assays, microhaematocrit centrifugation (MHC) and IFAT

Source	Checked	No. and (%) of animals:			
		PCR for <i>Trypanosoma vivax</i>	PCR for <i>Trypanozoon</i>	MHC	IFAT
Buffalo	316	60	0	36	121
Cow	15	3	0	1	6
Horse	6	0	0	0	3
Sheep	5	5	0	2	5
Any	342	68 (19.9)	0 (0)	39 (11.4)	135 (39.5)

The results of the parasitological and serological tests that were run in parallel with the PCR-based assays indicated that 39.5% (IFAT), 6.7% (microscopical examination of wet and dry bloodsmears) and 11.4% (MHC) of the samples were positive for trypanosomes or antibodies against *T. vivax* or *T. evansi*. The trypanosomes observed by microscopy were all too pleomorphic to be identified to species level from their morphology.

Although 29 of the 303 samples found negative by MHC were found positive by PCR, no samples were found negative by PCR and positive by MHC (Table 2). Therefore, with the results of the MHC used as reference, the PCR-based assay demonstrated 100% sensitivity and 90.4% specificity and gave a PPV of 0.57, a PLR of 10.45 and an NLR of 0.0 (Table 3).

DISCUSSION

The present research included the characterization and standardization of PCR-based assays for detecting trypanosomes in Venezuelan livestock and for differentiating the *T. vivax* infections from those of trypanosomes in the subgenus *Trypanozoon*. Such assays, which seem much more sensitive than MHC, could have a remarkable impact on the detection of 'animal' trypanosomiasis in Venezuela.

PCR based on the 21-mer/22-mer primer amplified some of the genomic DNA from all of the species in the subgenus *Trypanozoon* that were assessed. This

result was expected as Wuyts *et al.* (1995) indicated that the 21-mer and 22-mer oligonucleotides were specific not to a species but to the subgenus *Trypanozoon*. The present results support the theory that, irrespective of their current, often diverse, geographical distributions, all of the trypanosomes currently assigned to the subgenus *Trypanozoon* are phylogenetically related. This theory is also supported by the results of iso-enzymatic analyses, hybridizations with kinetoplast-DNA minicircle probes, and the analyses of kinetoplast DNA restriction-fragment-length polymorphisms (Lun *et al.*, 1992; Zhang and Baltz, 1994; Brun *et al.*, 1998). The nucleotide sequence of the Te-DNA probe used in the present study appears to be shared by most, if not all, species in the subgenus *Trypanozoon*. *Trypanosoma equiperdum*, *T. evansi* and *T. brucei* appear to be too closely related to be distinguished by standard PCR based on the repetitive sequences of their nuclear DNA, although Ventura *et al.* (1997) generated RAPD patterns that appeared to be specific for *T. evansi* from different hosts and countries. Since *T. evansi* appears to be the only species in the subgenus *Trypanozoon* that has been recognized in Venezuela or any other Latin American country, however, assays based on the 21-mer/22-mer primer pair could be considered species-specific (i.e. *T. evansi*-specific) in Latin America. Such assays can already detect just 10 pg of *T. evansi* DNA (Wuyts *et al.*, 1995; present study) and refinement of the DNA-isolation methodology and further optimisation of the PCR conditions (Desquesnes and Tresse,

TABLE 2. Comparison of the results from the PCR-based assays and microhaematocrit centrifugations (MHC), when the 342 blood samples from Venezuelan livestock were investigated

MHC result	No. of animals:		
	Positive in the PCR for <i>Trypanosoma vivax</i>	Negative in the PCR for <i>T. vivax</i>	Checked by PCR
Positive	39	0	39
Negative	29	274	303
Any	68	274	342

TABLE 3. The performance indicators of the PCR-based assay for the detection of *Trypanosoma vivax*, calculated using the results for the microhaematocrit centrifugation as the 'gold standard'

Indicator	Formula*	Value
Sensitivity (Se)	100TP/(TP+FN)	100.0%
Specificity (Sp)	100TN/(TN+FP)	90.4%
Positive predictive value	TP/(TP+FP)	0.57
Positive likelihood ratio	Se/(1-Sp)	10.45
Negative likelihood ratio	(1-Se)/Sp	0.0

*TP, Number of true positives; FN, number of false negatives; TN, number of true negatives; FP, number of false positives.

1996) could produce significant increases in sensitivity. The PCR-based assays developed, for the detection of trypanosomes, by Moser *et al.* (1989) and Clausen *et al.* (1998) had detection limits of several femtograms.

In the present study, the detection limit of the PCR based on the 21-mer/22-mer primer (about 10 pg of purified *T. evansi* DNA) represents approximately 50–100 trypanosomes/ml blood. A sample must usually contain many more trypanosomes to be found positive by the microscopical examination of wet or stained bloodsmears (10⁴ trypanosomes/ml), MHC (500–1000 trypanosome/ml), or any other, conventional, parasitological method (Paris *et al.*, 1982).

As Masake *et al.* (1997) also observed, the ILO1264/ILO1265 primer pair showed absolute species-specificity (no PCR products being generated with DNA from any species other than *T. vivax*) and gave an amplicon when the DNA template came from *T. vivax* collected in various areas of the world. The hybridization analyses demonstrated that the Tv-DNA probe was also highly specific for *T. vivax*. Even if based on *T. evansi* or *T. vivax* antigens, IFAT for the detection of anti-trypanosomal antibodies are often not very specific, and may give positive results with antibodies raised against a variety of *Trypanosoma* spp. and other trypanosomatids (Wells, 1984).

The IFAT investigated by Ferenc *et al.* (1990), for example, gave positive results with samples from animals that had been infected with *T. vivax*, *T. congolense*, *T. brucei* spp., *T. evansi* and, *T. equiperdum*. In Venezuela, however, *T. evansi* and *T. vivax* are the only salivarian trypanosomes that have been recorded in ungulates (and other common pathogens in ungulates, such as *Anaplasma marginale* and *Babesia* spp., do not seem to induce antibodies that cross-react with trypanosomal antigens; Ferenc *et al.*, 1990). Unwanted cross-reactions with antibodies induced by *T. cruzi* have been documented (Desquesnes and Tresse, 1999) but little is known about the prevalence of *T. cruzi* infection in livestock. The water buffaloes of Venezuela are perhaps more likely to be infected with another stercorearian species, *T. theileri*, than with *T. cruzi*. Antibodies against *T. theileri* do not, however, seem to interfere with ELISA or IFAT for detecting anti-*T. evansi* or anti-*T. vivax* antibodies in cattle (Desquesnes and Gardiner, 1993). Although *T. theileri* is a common and very wide-spread parasite of cattle, there are only a few reports of its occurrence in buffaloes, and no good data to prove that *T. theileri* from cattle and the *T. theileri*-like parasite in buffaloes are the same species (Rodríguez *et al.*, 2003).

Unfortunately, although bloodstream trypanomastigotes were observed in some of the samples of buffalo blood, they showed too much variability to be identified, morphologically, to species level; Toro *et al.* (1978) made a similar observation. If the veterinary significance of the trypanosomes in Venezuelan water buffaloes is to be accurately assessed by serology, the prevalence of *T. theileri* infection among these livestock, and the possibility that antibodies raised against the (presumably non-pathogenic) *T. theileri* in buffaloes cross-react with antigens from (potentially pathogenic) *T. evansi* or *T. vivax* in diagnostic IFAT or ELISA, need to be explored. The observation that far more of the livestock examined in the present

study were IFAT-positive than PCR-positive (139 *v.* 68) probably indicates the persistence of anti-trypanosomal antibodies in animals that are not currently infected.

Among the 342 animals (mostly buffaloes) checked in the present study, the prevalence of *T. vivax* infections indicated by the results of the PCR-based assays was almost 3- and 2-fold higher than the prevalence of trypanosome infection indicated by the microscopical evaluation of stained bloodsmears (6.7%) and MHC (11.4%), respectively. The PCR-based assays not only gave much better sensitivity than the other methods but also permitted the trypanosomes encountered in Venezuelan water buffaloes to be identified to species (all *T. vivax*), for the first time by molecular characterization. Surprisingly, none of the blood samples was found PCR-positive for *T. evansi*, not even those from the three horses found IFAT-positive. In Asia and Africa, *T. evansi* appears to be among the most common and pathogenic parasites of buffaloes (Löhr *et al.*, 1986; Luckins, 1988). The 29 MHC-negative but PCR-positive animals, all of which were also IFAT-positive, probably had relatively low *T. vivax* parasitaemias, which are characteristic of chronic infections in cattle (Rivera, 1996). Under laboratory conditions, using mice inoculated with *T. evansi*, Wuyts *et al.* (1995) found several MHC-negative animals to be positive in PCR based on the 21-mer/22-mer primer pair.

Holland *et al.* (2001) described how positive samples could appear negative by parasitological tests because they had been stored badly (i.e. for long periods and/or at warm temperatures) — samples with low parasitaemias often becoming negative after just 3 h of storage. In the present study, however, all the samples investigated were processed within the interval recommended by Paris *et al.* (1982) for the parasitological detection of *T. vivax*, *T. congolense* and *T. brucei* (6 h), and were only stored out of sunlight, at 4°C.

Otero *et al.* (2001) claimed that the predictive values normally calculated as performance indicators for a pathogen-detection assay were markedly affected by the local prevalence of the pathogen, and that likelihood ratios were more accurate indicators of the general usefulness of such assays. In the present study, even though the PPV for the assay based on the ILO1264/ILO1265 primer pair was low (0.57), the high PLR value (10.45) indicated that the assay results were trustworthy.

When Verloo *et al.* (2000) checked water buffaloes from North Vietnam for *T. evansi* infection, they only found 1.9% positive when they used a mouse-inoculation assay but 22% to be seropositive. Although this result probably again illustrates that anti-trypanosomal antibodies persist after parasitaemias have been cleared, it also indicates that *T. evansi* infection commonly occurs in North Vietnamese buffaloes. In the present study, similarly, almost 40% of the buffaloes investigated were found seropositive by IFAT. Compared with the IFAT or any other antibody-detection test, the PCR-based assay probably gives a much better idea of the current prevalence of infection. Assuming that *T. evansi* is very rare among the water buffaloes of Venezuela, the PCR-based assay for *T. vivax* (with the ILO1264/ILO1265 primer pair) could be used, probably as a supplement to the more conventional, parasitological and serological assays, to help determine the need for, and the effectiveness of, trypanosomiasis-control programmes for the buffaloes. Much more research on the epidemiology of trypanosome infection in the buffaloes of Venezuela is currently in progress.

ACKNOWLEDGEMENTS. This work was financially supported by grants S1-2001000988 and S1-2001000705 from the *Fondo Nacional de Ciencia, Tecnología e Innovación (Ministerio de Ciencia y Tecnología)* and grant PI:11-10-4832-01 from the *Consejo de Desarrollo Científico y Humanístico (Universidad*

Central de Venezuela). The authors are very grateful to Professors J. Rojas, T. Diaz and T. Sanchez (all of the *Universidad Central de Venezuela*) for improving their English. They particularly thank Professor H. Zerpa, for his critical reading of the manuscript. Many of the DNA samples used to develop the PCR-based assays were kindly donated by Drs F. Bringaud and A. M. Davila.

REFERENCES

Brun, R., Hermann, H. & Lun, Z.-R. (1998). *Trypanosoma evansi* and *Trypanosoma equiperdum*: distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitology*, **79**, 95–107.

Clausen, P. H., Wiemann, A., Patzelt, R., Kakaire, D., Poetzsch, C., Peregrine, A. & Mehlitz, D. (1998). Use of a PCR assay for the specific and sensitive detection of *Trypanosoma* spp. in naturally infected dairy cattle in peri-urban Kampala, Uganda. *Annals of the New York Academy of Science*, **849**, 21–31.

Desquesnes, M. & Gardiner, P. (1993). Épidémiologie de la trypanosomose bovine (*Trypanosoma vivax*) en Guyane Française. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, **46**, 463–470.

Desquesnes, M. & Tresse, L. (1996). Evaluation de la sensibilité de la PCR pour la détection de l'AND de *Trypanosoma vivax* selon divers modes de préparation des échantillons sanguins. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, **49**, 322–327.

Desquesnes, M. & Tresse, L. (1999). Utilization of *T. evansi* antigens in indirect-ELISA for diagnosis of Chagas disease in humans. In *Proceeding of First Symposium on New World Trypanosomes*, 20–22 November 1996, Georgetown, Guyana, eds Vokaty, S. & Desquesnes, M. pp. 111–114. Bridgetown, Barbados: Inter-American Institute for Cooperation on Agriculture.

Ferenc, S., Siopinskif, V. & Courtney, C. (1990). The development of an enzyme-linked immunosorbent assay for *Trypanosoma vivax* and its use in a seroepidemiological survey of the Eastern Caribbean Basin. *International Journal for Parasitology*, **20**, 51–56.

García, F. A. (1988). *Infección experimental de caballos con Trypanosoma evansi (=T. venezuelense). Aspectos patológicos y antigénicos*. M.Sc. thesis, Universidad Simón Bolívar, Caracas, Venezuela.

Holland, W. G., Claes, F., My, L. N., Thanh, N. G., Tam, P. T., Verloo, D., Büscher, P., Goddeeris, B. & Vercruyse, J. (2001). A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. *Veterinary Parasitology*, **97**, 23–33.

Katende, J. M., Musoke, A. J., Nantulya, V. M. & Goddeeris, B. M. (1987). A new method for fixation and preservation of trypanosomal antigen for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. *Tropical Medicine and Parasitology*, **38**, 41–44.

Kukla, B., Majiwa, P., Young, J., Moloo, S. & Ole-Moiyoi, O. (1987). Use of species-specific DNA probes for detection and identification of trypanosome infections in tsetse flies. *Parasitology*, **95**, 1–16.

Lanham, S. M. & Godfrey, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Experimental Parasitology*, **28**, 521–534.

Löhr, K., Pholpark, S., Siriwan, P., Leesirikul, N., Srikritjakarn, L. & Staak, C. (1986). *Trypanosoma evansi* infection in buffaloes in north-east Thailand. II. Abortions. *Tropical Animal Health and Production*, **18**, 103–108.

Luckins, A. G. (1977). Detection of antibodies in trypanosome-infected cattle by means of microplate enzyme-linked immunosorbent assay. *Tropical Animal Health and Production*, **9**, 53–52.

Luckins, A. G. (1988). *Trypanosoma evansi* in Asia. *Parasitology Today*, **4**, 137–142.

Lun, Z.-R., Brun, R. & Gibson, W. (1992). Kinetoplast DNA and molecular karyotypes of *Trypanosoma evansi* and *Trypanosoma equiperdum* from China. *Molecular and Biochemical Parasitology*, **50**, 189–196.

Masake, R. A., Majiwa, P. A., Moloo, S. K., Makau, J. M., Njuguna, J. T., Maina, M., Kabata, J., Ole-Moiyoi, O. K. & Nantulya, V. M. (1997). Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. *Experimental Parasitology*, **83**, 193–205.

McManus, D. & Bowles, J. (1996). Molecular genetic approaches to parasite identification: Their value in diagnostic parasitology and systematics. *International Journal for Parasitology*, **26**, 687–704.

Moser, D. R., Kirchoff, L. & Donelson, J. E. (1989). Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. *Journal of Clinical Microbiology*, **27**, 1477–1482.

Murray, M., Murray, P. K. & McIntyre, W. I. (1977). An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**, 325–326.

Nantulya, V. M. (1990). Trypanosomiasis in domestic animals: the problems of diagnosis. *Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux*, **9**, 357–367.

Obasi, O. L., Ogwu, D., Mohammed, G. & Okon, E. D. (1999). Reduced ovulatory and oestrus activity in zebu heifers following *Trypanosoma vivax* infection. *Tropical Animal Health and Production*, **31**, 55–62.

Otero, W., Pineda, L. & Beltrán, L. (2001). Utilidad de la razón de verosimilitud (likelihood ratio) en

- la práctica clínica. *Revista Colombiana de Gastroenterología*, **16**, 33–36.
- Paris, J., Murray, M. & McOdimba, F. (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica*, **39**, 307–316.
- Parra, D. & Vizcaino, O. (1976). *Manual de Técnicas del Programa de Parasitología y Entomología Veterinaria*. Bogota: Instituto Colombiano Agropecuario.
- Rivera, M. A. (1996). *Hemoparasitosis Bovinas*. Caracas, Venezuela: Anaco Ediciones.
- Rodrigues, A., Campaner, M., Takata, C., Dell' Porto, A., Milder, R., Takeda, G. & Teixeira, M. (2003). Brazilian isolates of *Trypanosoma (Megatrypanum) theileri*: diagnosis and differentiation of isolates from cattle and water buffalo based on biological characteristics and randomly amplified DNA sequences. *Veterinary Parasitology*, **116**, 185–207.
- Smith, G. & Summer, M. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. *Analytical Biochemistry*, **109**, 123–129.
- Thrusfield, M. (1990). *Epidemiología Veterinaria*. Zaragoza, Spain: Editorial Acribia.
- Toro, M., León, E., López, B., García, J. A. & Ruiz, A. (1978). *Informe Técnico sobre Hemoprotozoarios en Búfalos, Équidos y Bovinos en Isla de Güara, Estado Monagas*. Maracay, Venezuela: Instituto de Investigaciones Veterinarias.
- Ventura, R. M., Takeda, G. F. & Teixeira, M. M. (1997). Molecular markers for characterization and identification of *Trypanosoma evansi*. In *Proceeding of the First Internet Conference on Salivarian Trypanosomes* (FAO Animal Production and Health Paper No. 136), pp. 25–28. Rome: Food and Agriculture Organization.
- Verloo, D., Holland, W., My, L. N., Thanh, N. G., Tam, P. T., Goddeeris, B., Vercruyse, J. & Büscher, P. (2000). Comparison of serological test for *Trypanosoma evansi* natural infections in water buffaloes from North Vietnam. *Veterinary Parasitology*, **92**, 87–96.
- Voller, A., Bidwell, D. E. & Bartlett, A. A. (1975). Serological study on human *Trypanosoma rhodesiense* infections using a micro-scale enzyme-linked immunosorbent assay. *Tropenmedizin und Parasitologie*, **26**, 247–251.
- Wells, E. A. (1984). Animal trypanosomiasis in South America. *Preventive Veterinary Medicine*, **2**, 31–41.
- Woo, P. T. K. (1969). The haematocrit centrifuge for the detection of trypanosomes in blood. *Canadian Journal of Zoology*, **47**, 921–923.
- Wuyts, N., Chokesajjawatee, N., Sarataphan, N. & Panyin, S. (1995). PCR amplification of crude blood on microscope slides in the diagnosis of *Trypanosoma evansi* infection in dairy cattle. *Annales de la Société Belge de Médecine Tropicale*, **75**, 229–237.
- Zhang, Z. & Baltz, T. (1994). Identification of *Trypanosoma evansi*, *Trypanosoma equiperdum* and *Trypanosoma brucei brucei* using repetitive DNA probes. *Veterinary Parasitology*, **53**, 197–208.