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Serodiagnosis of bovine trypanosomosis caused by non-tsetse transmitted *Trypanosoma* (Duttonella) *vivax* parasites using the soluble form of a Trypanozoon variant surface glycoprotein antigen

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ABSTRACT

Previous studies have shown that a 64-kDa antigen (p64) that was purified from the Venezuelan TeAp-N/D1 isolate of *Trypanosoma* (Trypanozoon) *equiperdum* corresponds to the soluble form of its predominant variant surface glycoprotein (VSG), and exhibited cross-reactivity with *Trypanosoma* (Duttonella) *vivax*. The course of experimental acute infections of bovines with *T. vivax* were followed by measuring whole anti-p64 antibodies and specific anti-p64 IgG and IgM antibodies in animal sera by indirect enzyme-linked immunosorbent assay (ELISA). The value of p64 to diagnose bovine trypanosomosis was also examined using 350 sera from healthy and *T. vivax*-infected cows living in a trypanosomosis-endemic and enzootic stable area, and 48 sera obtained during a trypanosomosis outbreak. Serological assays showed that ~70–80% of the infected sera contained anti-p64 antibodies, based on the comparative immunodetection of the *T. equiperdum* clarified antigenic fraction used as a reference test. In the absence of a gold standard, Bayesian analysis for multiple testing estimated a sensitivity and specificity of 71.6% and 98.8%, respectively, for the indirect ELISA using p64 as antigen. An apparent prevalence of 37.7% for bovine trypanosomosis infection was also estimated with a Bayesian approach when the p64 ELISA test was used. Employing blood from acute infected cows, the indirect ELISA response against p64 was contrasted with the microhematocrit centrifuge method and analyses by polymerase chain reaction (PCR) using specific primers targeting the inter-specific length variation of the internal transcribed spacer 1 region of the 18S ribosomal gene. The efficiency of p64 for the detection of anti-trypanosome antibodies in acute infected bovines was also corroborated serologically by comparing its response to that of the Indonesian *Trypanosoma evansi* Rode Trypanozoon antigen type (RoTat) 1.2 VSG, which possesses high specificity and sensitivity. As expected, PCR was the best method to detect parasites and diagnose bovine trypanosomosis; however, a substantial level of concordance (Cohen's $\kappa = 0.667$) was obtained when serological tests using p64 and RoTat 1.2 VSG were compared. Additionally, an agglutination assay was designed using p64 covalently coupled to carboxylate-modified latex microparticles, which was proven here to be suitable for a fast qualitative diagnosis of bovine trypanosomosis.

Abbreviations: VSG, variant surface glycoprotein; VAT, variant antigen type; RoTat, RodeTrypanozoon antigen type; ELISA, enzyme-linked immunosorbent assay; MHC, microhematocrit centrifuge; PCR, polymerase chain reaction; ITS1, internal transcribed spacer 1 region; MPP, microparticles; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; PCV, packed cell volume; HAT, human african trypanosomiasis; DME, direct microscopic examination; IIF, indirect immunofluorescence.

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1. Introduction

In Venezuela, *Trypanosoma* (Duttonella) *vivax* is the major causative agent of trypanosomosis in cattle (Rivera, 1996). *T. vivax* is a non-tsetse transmitted trypanosome in this region, and is spread mechanically by bloodsucking flies such as *Tabanidae* and *Stomoxys* species (Desquesnes, 2004; Osório et al., 2008). Bovine trypanosomosis causes severe anemia, edema, immunosuppression, and various neurological disorders, which may eventually produce the death of the affected animals (Gonzatti et al., 2014). Hence, bovine trypanosomosis generates significant economic losses to the farmers in terms of morbidity, mortality, abortion, infertility, reduced milk yield, and costs for trypanocides.

Salivarian parasites evade the adaptive immune system of the host using an antigenic variation strategy (Horn, 2014; Cnops et al., 2015). The surface of salivarian trypanosomes is covered with a densely packed layer of dimers of one type of variant surface glycoprotein (VSG). These VSGs are strong immunogens, but the parasite avoids elimination by the host immune system by changing the variant antigen type (VAT) of its VSG coat. Each parasite genome contains a large repertoire of several hundred to thousand *VSG* genes, supplemented by recombination and gene conversion events; however, only one is predominantly expressed at a time (Berriman et al., 2005; McCulloch and Horn, 2009). Switching the expression of one *VSG* gene to another results in a trypanosome bearing a different VAT, that may escape immune destruction as long as the infected host do not possess antibodies against this particular VAT (Barry and McCulloch, 2001). This antigenic variation strategy makes it difficult to develop a VSG-based vaccine against animal trypanosomosis, and vaccine design strategies have been focused on invariant trypanosome molecules that mediate pathogenesis.

Despite the switching strategy of *VSG* genes, various reports have shown that native and recombinant VSG antigens, VSG peptides and VSG mimotopes can be used for the diagnosis of salivarian trypanosomes (Bajyana Songa and Hamers, 1988; Ngaira et al., 2004; Penchenier et al., 2003; Sengupta et al., 2012; Van Nieuwenhove et al., 2012, 2013). Particularly, serological and PCR tests based on the VSG of *Trypanosoma evansi* Rode Trypanozoon antigen type (RoTat) 1.2, a VAT derived from an Indonesian stock of *T. evansi* isolated from a buffalo in 1982, have shown high specificity and sensitivity (Bajyana Songa and Hamers, 1988; Claes et al., 2004; Urakawa et al., 2001; Verloo et al., 2000). We have purified to homogeneity a 64-kDa glycosylated antigen (p64) from the Venezuelan TeAp-N/D1 strain of *Trypanosoma* (Trypanozoon) *equiperdum* (*aka* TEVA1), which corresponded to the soluble form of its predominant VSG and appeared to be very sensitive for diagnostic purposes (Uzcanga et al., 2004). Interestingly, p64 was also recognized by anti-*T. vivax* bovine antibodies (Uzcanga et al., 2002, 2004). TeAp-N/D1 was previously considered as a *T. evansi* isolate (Espinoza et al., 1997; Uzcanga et al., 2002, 2004; Camargo et al., 2004; Velásquez et al., 2014); however, Sánchez et al. (2015) have recently demonstrated that TeAp-N/D1 belongs to the *T. equiperdum* species by its molecular characterization using microsatellite markers and kinetoplast maxicircle genes. We have also purified and characterized the soluble forms of six additional VSGs from Venezuelan animal trypanosomes (Camargo et al., 2015). Like p64, all purified soluble VSGs exhibited cross-reactivity with *T. vivax* and were able to be used as diagnostic reagents for bovine trypanosomosis (Camargo et al., 2015). The purpose of this study was to evaluate the efficacy of p64 for the serodiagnosis of cattle experimentally or naturally infected with *T. vivax*.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following sources: anti-bovine IgG (whole molecule) horseradish peroxidase conjugate, anti-bovine IgG (whole molecule) alkaline phosphatase conjugate, fluorescein-conjugated anti-bovine IgG (whole molecule), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, Sigma; horseradish peroxidase labeled anti-bovine IgG (γ) polyclonal secondary antibody, horseradish peroxidase labeled anti-bovine IgM (μ) polyclonal secondary antibody, KPL; Wizard® DNA extraction Kit, 5-bromo-4-chloro-3 indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), Promega; SYBR® Safe DNA gel stain, Invitrogen; bicinchoninic acid BCA™ Protein Assay Kit, nitrocellulose (0.45 μm pore size), Pierce; 96-well polypropylene plates (PolySorp or MaxySorp), Nunc; Opti-Link carboxylate-modified polystyrene (latex) microparticles (MPP), Seradyn; 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), Sigma or Roche; ABTS-buffer (phosphate-citrate-sodium perborate solution, pH 4.6), Roche. All other chemicals were of the highest quality grade available.

2.2. Preparation of the clarified antigenic fraction from the TeAp-N/D1 T. equiperdum isolate

T. equiperdum parasites ($\sim 10^9$) from the Venezuelan TeAp-N/D1 isolate were extracted on ice by sonication using 2 ml of a 5 mM Tris–HCl buffer (pH 7.2) containing 1 mM benzamidine, 1 mM phenyl methyl sulfonyl fluoride, 5 mM EDTA, and 1 mM iodoacetamide. The resulting homogenate was centrifuged at 15000 × *g* for 30 min, at 4 °C, to obtain the supernatant and pellet fractions. The supernatant fraction was defined as the clarified antigenic fraction from *T. equiperdum*, and was used as the source of parasite antigens for both indirect ELISA and Western blot analyses.

2.3. Purification of p64 from the TeAp-N/D1 T. equiperdum isolate

The p64 antigen was purified from the TeAp-N/D1 *T. equiperdum* isolate following the procedure described by Uzcanga et al. (2002, 2004).

2.4. Blood samples from field animals

For the diagnosis of bovine trypanosomosis, blood samples were collected from the jugular vein of 350 cows from a group of beef production farms located in the Monagas Municipality, Guárico State, which is a trypanosomosis-endemic and enzootic stable area in Venezuela. These 350 bovines were asymptomatic and possessed normal blood parameters, which suggested that they were apparently healthy. Sera from 48 hybrid Brahman cows were also obtained from a farm located near Caicara del Orinoco, Bolívar State, Venezuela, during a trypanosomosis outbreak. These 48 bovines contained numerous *T. vivax* parasites in their blood and showed clinical symptoms such as neurological signs, atypical weight, moderate or severe anemia, and decreased milk production.

Consent was obtained from all owners of the animals analyzed in this study, and all precautions were taken to minimize any contamination and suffering.

2.5. Experimental infection of animals

The *T. vivax* LIEM-176 isolate employed here was acquired from a naturally infected bovine from the Trujillo State, Venezuela (Gómez-Piñeres et al., 2014). Inoculating cryopreserved bovine

infected blood into goats allowed further expansions of *T. vivax* LIEM-176, which was subsequently purified using Percoll gradients (Grab and Bwayo, 1982). Two hybrid Siboney bovines that were healthy and negative to trypanosomosis by the classical parasitological microhematocrit centrifuge (MHC) test (Woo, 1970), and various serological techniques such as indirect immunofluorescence (IIF), indirect ELISA, and Western blot (Uzcanga et al., 2002), were experimentally infected with $\sim 10^6$ *T. vivax* parasites. Blood samples from experimentally infected cows, containing heparin as anticoagulant, were taken every day for approximately a two-month period in order to determine the parasitaemia by the MHC method. The clinical condition of each infected animal was determined by measuring its hematocrit [volume percentage of erythrocytes in blood or packed cell volume (PCV)] and body temperature. Rectal temperatures were taken using a digital thermometer. During the entire course of the experiments, safety measures were employed to diminish any distress of the experimentally-infected animals. When their physical conditions deteriorated, the infected cows were treated with a curative dose of isometamidium chloride (0.5 mg/Kg).

2.6. Bovine antibody responses against p64 by indirect ELISA

Indirect ELISA was carried out according to the method described by Uzcanga et al. (2002). Briefly, ELISA plates (PolySorp, Nunc) were sensitized with 160 ng of the purified p64 antigen/well in carbonate/bicarbonate buffer (pH 9.6). Following an overnight incubation at 4 °C using a humid chamber, an excess of blocking buffer A (0.02 M sodium phosphate, pH 7.2, 0.15 M NaCl, 0.1% v/v Tween 20, 5% w/v skimmed milk) was applied to each well for 1 h, at 37 °C. Sera from experimentally or naturally infected animals were diluted 1:400, and an aliquot of 100 μl was added per well. After an extensive wash, a horseradish peroxidase-conjugated secondary antibody against bovine IgG (dilution 1:2000) was supplemented (100 μl/well). The color reaction was developed during 1 h, employing 200 μl of a solution prepared with 10 mg of ABTS in 100 ml of 0.05 M phosphate/citrate buffer (pH 5) and 0.0075% hydrogen peroxide. Optical densities were read at 405 nm.

A similar procedure was utilized to measure the anti-p64 γ-specific IgG and μ-specific IgM antibody response during the course of the experimental infection of bovines with *T. vivax*. In these cases, affinity purified and horseradish peroxidase tagged polyclonal antibodies against bovine γ-specific IgG (dilution 1:100), or bovine μ-specific IgM (dilution 1:100), were employed as the secondary antibodies following the specifications of the vendor.

For the determination of the cut off values, sera from 30 healthy bovines living in France, which is a trypanosomosis non-endemic area, were evaluated as negative controls. All these reference bovine sera were analyzed by indirect ELISA, in order to establish a confidence interval based on the normal distribution of the optical density (De Savigny and Voller, 1980). The cut off value was calculated by the variance method as the mean absorbance value obtained from the negative sera plus three standards deviations.

2.7. Purification of VSG from T. evansi RoTat 1.2

Native RoTat 1.2 VSG was purified following the procedure described by Magnus et al. (1978), and was kindly donated by Dr. Philippe Büscher, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.

2.8. PCR analysis

For PCR evaluation, we used the collection of bovine blood samples acquired during a trypanosomosis outbreak in the Caicara del Orinoco farm, Bolívar State, Venezuela. Parasite genomic DNA was obtained using the Wizard® DNA extraction Kit, according to the instructions of the manufacturer. PCR analyses were carried out using specific primers targeting the ITS1 region of the 18S ribosomal gene, which has been reported to differ among trypanosome species (Njiru et al., 2005). As positive controls, DNA was extracted from the Venezuelan TeAp-El Frio01 *T. evansi* and LIEM-176 *T. vivax* isolates. DNA obtained from the African IL3000 *Trypanosoma* (Nannomonas) *congolense* isolate was also included as a reference. PCR results were compared to those obtained by the MHC test, and by indirect ELISA using either p64 or the RoTat 1.2 VSG as antigens. SYBR® Safe staining was employed for visualization of DNA in agarose gels.

2.9. Comparison of bovine antibody responses against p64 or the RoTat 1.2 VSG by Indirect ELISA

MaxySorp plates (Nunc) were coated overnight at 4 °C with 100 μl/well of p64 (8 μg/ml) or native RoTat 1.2 VSG (1 μg/ml) in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4). Plates were blocked for 1 h, at room temperature, with 350 μl/well of 0.01 M sodium phosphate (pH 7.4), 0.2 M NaCl, 0.05% v/v Tween 20, 1% w/v skimmed milk, and 0.05% w/v NaN_3 (blocking buffer B). Bovine sera were diluted 1:500 in blocking buffer B, and 100 μl of each bovine serum was added to the wells in quadruplicates. Following an incubation for 1 h at room temperature, the plates were washed nine times with 350 μl/well of PBS containing 0.05% v/v Tween 20 (pH 7.4) using an automated plate washer (Elx50, BIO-TEK). An aliquot (100 μl/well) of horseradish peroxidase-conjugated secondary antibodies against bovine IgG (dilution 1:10000) was supplemented, and incubated for 1 h, at room temperature. After five washes, chromogenic reactions were developed using 100 μl/well of 0.5 mg/ml ABTS in ABTS-buffer (pH 4.6). Absorbance was read at 414 nm using a Multiskan RC, Version 6.0 (Labsystems). Corrected OD values were obtained by subtracting the mean OD of the antigen-containing wells from the mean OD of the corresponding antigen-free control wells.

2.10. Agglutination test using the p64 antigen adsorbed and covalently coupled to carboxylate-modified latex microparticles

In order to optimize the amount of bound p64, increasing concentrations of p64 were mixed with 1% w/v carboxylate-modified polystyrene microparticles (MPP) in 50 mM Mes (pH 6.1), in the presence of a 2.5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide over particle carboxyl concentration (Goodfriend et al., 1964; Kondo et al., 1992). Following an overnight incubation at room temperature, a glycine solution was added (final concentration of 1.0 M), and the reaction was carried out for 2 h to block the excess carbodiimide. The reaction mixtures were then centrifuged for 15 min, at 15000 rpm (Eppendorf centrifuge), to separate the supernatants containing the non-adsorbed protein. The pellets with the sensitized MPP were washed twice in 50 mM Mes (pH 6.1), and resuspended in the initial volume using the same buffer. The concentration of bound and non-bound p64 was determined employing a BCA™ Protein Assay Kit according to the instructions of the manufacturer, and a curve of the amount (μg) of bound p64 per mg of latex MPP was generated versus the amount (μg) of added p64 per mg of latex MPP. The optimal p64 concentration was then used to sensitize the batch of MPP utilized for the agglutination test. The p64-sensitized latex particles were blocked with 1% w/v BSA in 25 mM Tris (pH 8.4), 100 mM NaCl, and 0.1% w/v NaN_3 for at least 1 h at room temperature, before use. Various dilutions of reference sera from bovines naturally or experimentally infected with trypanosomes were initially used to determine the appropriate dilution in the assay. We found that sera dilutions

between 1:64 and 1:128 were optimal. As controls, MPP free of p64 were incubated with sera from bovines parasitologically and serologically positive to trypanosome, and p64-bound MPP were also incubated with reference sera from bovines parasitologically and serologically negative to trypanosome. To standardize the agglutination assay, a total of 60 bovine sera (dilution = 1:120) were used to evaluate the test. These sera included 20 and 40 samples that were identified as negative and positive, respectively, by both an indirect ELISA using p64 as antigen and the classical parasitological MHC assay (Woo, 1970).

2.11. Other procedures

The protein concentration was determined using bovine serum albumin as protein standard (Bradford, 1976). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on 1.5-mm thick slab gels containing 12% (w/v) polyacrylamide as described by Laemmli (1970). Coomassie blue R-250 staining was used for protein visualization. For Western blot analyses, proteins were transferred from the gels to nitrocellulose sheets as described by Towbin et al. (1979). The nitrocellulose filters were incubated with bovine sera (dilution 1:100), and the membranes were then treated with the appropriate dilution of alkaline phosphatase-conjugated secondary antibodies against bovine IgG. Polypeptide bands were visualized by the addition of NBT and BCIP, according to the manufacturer. Trypanosomes were detected by direct microscopic examination (DME) (Brener, 1962) and the MHC test (Woo, 1970). For DME, wet smears of fresh bovine blood (5 μl) were examined by direct observation of 100 fields using a phase contrast microscope. For MHC, fresh bovine blood (75 μl) was taken with a heparinized capillary and the tubes were examined using a light microscope. Motile trypanosomes were detected near the buffy coat. IIF was carried out following the procedure described by Cons and Kaplan (1950). Microscope slides pre-coated with blood of *T. equiperdum*-infected Sprague Dawley rats were incubated with different dilutions of bovine sera. Then, a fluorescein-conjugated secondary antibody against bovine IgG (dilution 1:50) was employed. The parasites were viewed using a fluorescence microscope directly connected with a photographic camera.

2.12. Statistical analysis

The sensitivity and specificity for the indirect ELISA using the purified p64 antigen was initially calculated by using the indirect ELISA for the *T. equiperdum* clarified antigenic fraction as a reference test. In addition, a Bayesian statistical modeling was employed because none of the diagnostic tests included in the present study was a gold standard. Bayesian analysis allowed us to estimate the sensitivity and specificity for the indirect ELISA using p64 as antigen, on the basis of a population sample of 350 cows that were surveyed in a trypanosomosis-endemic area in Venezuela. Prior information about test characteristics on DME, MHC and indirect ELISA using the parasite clarified fraction was extracted from available literature reporting either *T. evansi* (Ramírez-Iglesias et al., 2011; Reyna-Bello et al., 1998) or *T. vivax* infections (Eisler et al., 1998). Our initial determination of the sensitivity and specificity for the p64 indirect ELISA was also included in the analysis. A multinomial Bayesian model adapted from Berkvens et al. (2006) was employed by using conditional probabilities of the four tests, and the analysis was performed using the software R "Prevalence" package for multiple testing (Praet et al., 2010, 2013).

The level of agreement between diagnostic tests on the 48 cows from Caicara del Orinoco was determined using Cohen's kappa coefficient (κ) interpreted following Landis and Koch (Cohen, 1960; Landis and Koch, 1977). Cohen's κ is a statistical measure of inter-rater agreement or concordance that takes into account the agreement occurring by chance. If the raters are in complete agreement then $\kappa = 1$. On the other hand, $\kappa = 0$ if there is no agreement among the raters other than what would be expected by chance. Hence, values ≤ 0 indicate no agreement; values between 0.020 and 0.4 show a fair agreement, values in the 0.61–0.80 range show a substantial agreement, and values above 0.80 represent an almost perfect agreement (Landis and Koch, 1977).

3. Results

3.1. Antibody responses against p64 during the experimental acute infection of bovines with T. vivax

Former reports from our laboratory have revealed that p64 is a *T. equiperdum* antigen that exhibited cross-reactivity with *T. vivax* (Uzcanga et al., 2002, 2004; Camargo et al., 2015). These findings have shown that p64 contains common epitopes recognized by sera from animals infected either with Trypanozoon trypanosomes or *T. vivax*. Then, the amount of anti-p64 antibodies in sera was measured by indirect ELISA during the course of the experimental infection of two bovines with *T. vivax*. Measurements performed with bovines 1 and 2 are illustrated in Fig. 1A and B, respectively.

Initially, curves of parasitaemia, hematocrit, and temperature for the *T. vivax*-infected bovines were evaluated. Parasites were not detected during the prepatent period, which lasted until day 7 post infection in both cows (Fig. 1A and B, upper panels). When the experiment started, bovines 1 and 2 had PCV values of 28 and 30, respectively, and rectal temperatures of 38.0–38.5 °C (Fig. 1A and B, lower panels). Consistently, PCV and temperature values remained in their normal range during the prepatent period. The first peak of parasitaemia started at day 8 post infection in both bovines (Fig. 1A and B, upper panels). Then, the level of parasitaemia showed regular fluxes (rises and falls), corresponding to the characteristic parasitaemia waves that indicate the emergence, expansion and removal of antigenically distinct populations of parasites containing different VATs (Fig. 1A and B, upper panels). One of the main hematological changes observed in natural cases of bovine trypanosomosis is anemia associated with decrease in PCV. As shown in Fig. 1A and B (lower panels), both bovines showed a reduction in PCV following infection, and at the end of the experiment, the hematocrit dropped to values of 18 and 20 for bovines 1 and 2, respectively. In addition, the rectal temperature of both animals started rising from day 7 after infection, and then fluctuated throughout the whole study period. The mean rectal temperatures of infected cows 1 and 2 were 38.9 °C ± 0.80 and 39.2 °C ± 0.7, respectively (Fig. 1A and B, lower panels), which were higher than the normal temperatures reported for healthy bovines. The highest temperature recorded was 42 °C and 41.5 °C for bovines 1 and 2, respectively. On the basis of the development of clear signs of acute infections with *T. vivax*, such as the appearance of parasites in their blood, emergence of recurrent fever that coincided with the parasitaemia waves, and manifestation of a descent of their hematocrit (Gonzatti et al., 2014), we concluded that these infected bovines were in the acute period of infection. Moreover, a deterioration of the cows' physical state was detected, such as progressive weight loss and lethargy, which persisted until the animals were treated with remedial amounts of isometamidium chloride.

Both experimentally infected cows showed a curve response characterized by the appearance of a maximum in total IgG anti-p64 antibody production on day 10 after infection (Fig. 1A and B, upper panels). Interestingly, high levels of complete anti-p64 IgG antibodies were detected in sera from these bovines during the whole course of the acute infection. Anti-p64 μ-specific IgM and γ-specific IgG antibody production was also determined in the experimen-

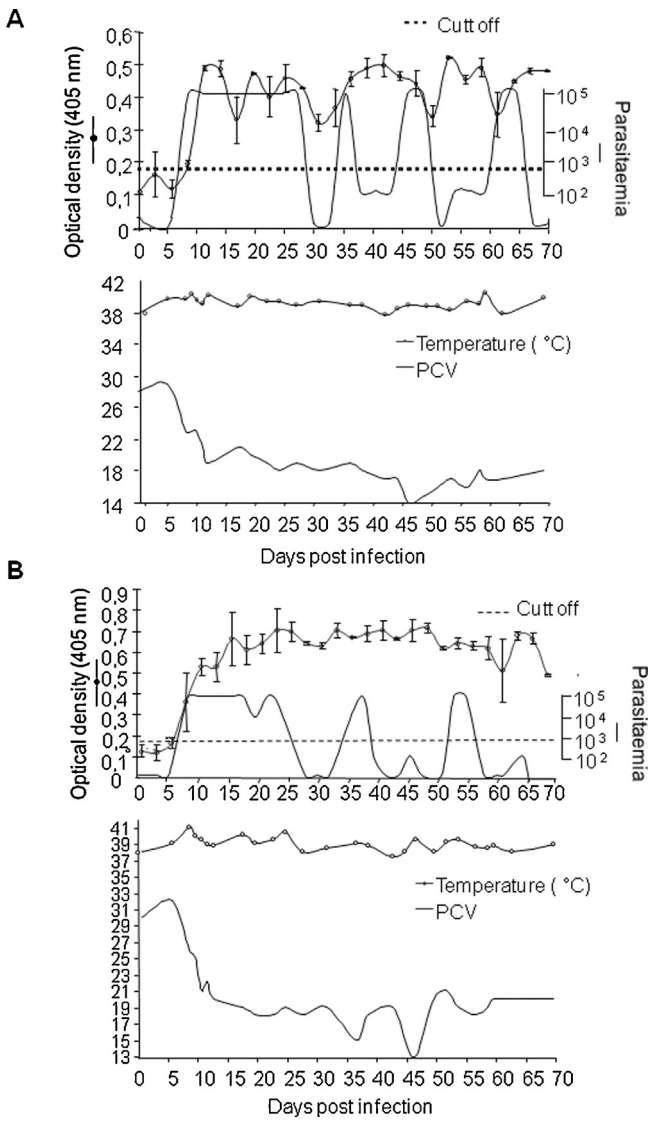

Fig. 1. Antibody responses against p64 during the experimental infection of two bovines with *T. vivax*. Two healthy cows were experimentally infected with the *T. vivax* LIEM-176 Venezuelan isolate (A and B). Production of anti-p64 antibodies was evaluated by indirect ELISA (■), and was correlated with the time course of parasitaemia (continuous line, upper panels), body temperature (●) and hematocrit (PCV; continuous line, lower panels). Cut off values for p64 are shown (- - -).

tally infected bovines. As shown in Fig. 2 for bovine 2, an increase in the generation of anti-p64 μ-specific IgM and γ-specific IgG antibodies started at day 10 after infection, and the levels of both isotypes fluctuated during the whole course of the experiment. A maximum in the amount of anti-p64 μ-specific IgM production was sustained from day 10 post infection to the end of the time course, during most of the experiment (Fig. 2A). However, the OD values of anti-p64 μ-specific IgM antibodies dropped below its corresponding cut off value between days 48 and 51 post infection (Fig. 2A). A peak in the generation of anti-p64 γ-specific IgG antibodies was observed at about 3–4 weeks after infection, which persisted until the end of the experiment (Fig. 2B). Yet, the OD values of anti-p64 γ-specific IgG antibody were kept above its corresponding cut off value during the whole time course of the infection, even during days 48 and 51 post infection (Fig. 2B), accounting for the high levels of complete anti-p64 IgG antibodies that were detected in sera from this bovine during the whole course of the acute infection (Fig. 1B). Comparable results were attained when sera from bovine 1 were used (data not shown). These results (Figs. 1 and 2)

Table 1
Results of seven diagnostic tests applied on 350 bovines from farms located in a trypanosomosis-endemic and enzootic stable area in Venezuela.

DME	MHC	IIF	CF ELISA	CF WB	p64 ELISA	p64 WB	No. of animals
1	1	1	1	1	1	1	62
0	1	1	1	1	1	1	49
0	0	1	1	1	1	1	13
0	0	1	1	1	0	1	11
0	0	1	1	1	0	0	41
0	0	1	0	0	0	0	10
0	0	0	1	1	1	1	8
0	0	0	1	1	0	1	2
0	0	0	0	0	0	0	154

0 = negative test result; 1 = positive test result. No. of animals, number of cows for each result category. DME, direct microscopic examination; MHC, microhematocrit centrifuge test; IIF, indirect immunofluorescence; CF ELISA, indirect ELISA using the *T. equiperdum* clarified antigenic fraction; CF WB, Western blot using the *T. equiperdum* clarified antigenic fraction; p64 ELISA, indirect ELISA using the purified p64 antigen from *T. equiperdum*; p64 WB, Western blot using the purified p64 antigen from *T. equiperdum*.

showed that p64 can be used as a potentially good antigen to detect antibodies to *T. vivax* parasites for immunodiagnosis during acute infections.

3.2. The p64 antigen was immunorecognized by sera obtained from T. vivax-positive cows

Blood samples from 350 bovines were collected and inspected for trypanosomes by DME of wet blood films, and the MHC test (Table 1). *T. vivax* parasites were identified since they presented a distinctive motion consisting of a rapid vibrational movement often followed by speedy translational movement across the field of view (Bruce et al., 1910). A group of 62 animals showed infection with *T. vivax* by DME. Additionally, the parasites in the cryopreserved blood samples did not expand into adult albino rats as reported for other Venezuelan *T. vivax* isolates (Gómez-Piñeres et al., 2009). A total of 111 cows, that included the 62 diagnosed by DME, contained trypanosomes by the MHC technique. On the basis of the high cross-reactivity reported between Trypanozoon trypanosomes and *T. vivax*, sera from the 350 bovine blood samples were also evaluated by IIF using fixed *T. equiperdum* TeAp-N/D1 parasites, and by indirect ELISA and Western blot using either the *T. equiperdum* TeAp-N/D1clarified antigenic fraction or the purified p64 (Table 1). We found that 186 bovine sera reacted positively against the clarified antigenic fraction of *T. equiperdum* by both indirect ELISA and Western blot. When p64 was employed as antigen, 71% (132 sera) and 78% (145 sera) of this group of 186 positive sera recognized p64 by indirect ELISA and Western blot, respectively. In addition, a group of 186 sera was found to recognize *T. equiperdum* by IIF microscopy; however, only 176 of the IIF positive samples were diagnosed as positive by either indirect ELISA or Western blot using the *T. equiperdum* clarified antigenic fraction, yielding 10 sera samples that probably gave nonspecific results by IIF.

By defining the true positives and true negatives based on the detection of the *T. equiperdum* clarified antigenic fraction by the sera samples as a reference test, we were able to calculate the sensitivity and specificity for the indirect ELISA test when p64 was used as antigen. Values of sensitivity and specificity of 77.8% and 88%, respectively, were achieved, and a positive predictive value of 70% was calculated for the p64 indirect ELISA test.

Although seven different tests were used to examine the 350 bovine blood samples (Table 1), prior information on sensitivity and specificity was readily available for only three of these tests: DME, MHC and indirect ELISA using the parasite clarified fraction (Ramírez-Iglesias et al., 2011; Reyna-Bello et al., 1998; Eisler et al., 1998). Employing these values, together with the information

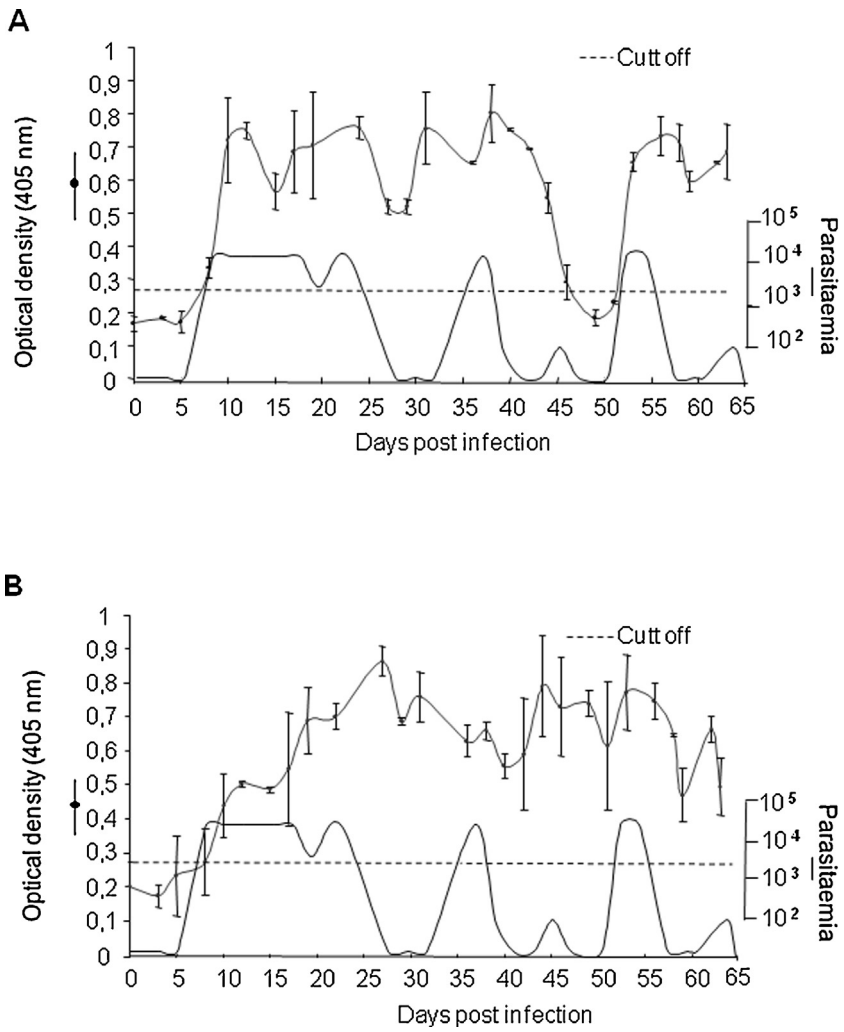

Fig. 2. Time course of anti-p64 μ-specific IgM and γ-specific IgG antibody production in a cow experimentally infected with the *T. vivax* LIEM-176 isolate. Generation of specific anti-p64 IgM (A) and IgG (B) antibodies in sera during the course of the infection was evaluated by indirect ELISA (■), and was related with the parasitaemia (continuous line). Cut off values for p64 are shown (- - -).

Table 2
Bayesian estimates of the characteristics of four diagnostic tests used for the detection of *T. vivax*-infected animals, on a population of 350 cows from farms located in a Venezuelan trypanosomosis-endemic region.

Diagnostic test	Sensitivity (95% CI)	Specificity (95% CI)
DME	0.346 (0.286–0.406)	0.972 (0.935–0.993)
MHC	0.601 (0.553–0.644)	0.978 (0.943–0.996)
CF ELISA	0.808 (0.705–0.881)	0.832 (0.414–0.998)
p64 ELISA	0.716 (0.711–0.729)	0.988 (0.954–1.000)

CI = credibility interval; DME, direct microscopic examination; MHC, microhematocrit centrifuge test; CF ELISA, indirect ELISA using the *T. equiperdum* clarified antigenic fraction; p64 ELISA, indirect ELISA using the purified p64 antigen from *T. equiperdum*.

obtained above for the indirect ELISA test using p64 as antigen, the sensitivity and specificity values for the p64 indirect ELISA were recalculated by a Bayesian approach. Bayesian estimation of the sensitivity and specificity of the other diagnostic methods for the detection of the disease (DME, MHC, and indirect ELISA using the *T. equiperdum* clarified fraction) showed no disagreement with the information previously published (Ramírez-Iglesias et al., 2011; Reyna-Bello et al., 1998; Eisler et al., 1998). As shown in Table 2, the lowest sensitivity was achieved by DME, followed by the MHC technique, indirect ELISA using p64 as antigen, and indirect ELISA using the *T. equiperdum* clarified fraction. However, the specificity of the p64 indirect ELISA was higher than the specificity of the indirect ELISA using the *T. equiperdum* clarified fraction given that the latter had a very wide range of credibility (Table 2). Summarizing, sensitivity and specificity values of 71.6% and 98.8%, respectively, were calculated by a Bayesian approach for the indirect ELISA using p64 as antigen. After combining the four independent diagnostic methods applied to the same population sample, the Bayesian analysis estimated an apparent prevalence of 37.7% (95% CI: 32.7–42.9%) when the p64 ELISA test was used.

Given that these sera samples were collected in a Venezuelan trypanosomosis-endemic area, and although some sporadic outbreaks might also occur in enzootic stable regions, we presumed that most of these trypanosomosis diagnosed bovines were probably chronically sick animals. Therefore, we also propose that p64 can be employed as a good quality antigen to detect anti-*T. vivax* antibodies for immunodiagnosis during chronic infections of cattle.

3.3. Diagnosis of bovine trypanosomosis by the MHC technique, PCR, indirect ELISA using p64 and indirect ELISA using RoTat 1.2 VSG

During a bovine trypanosomosis outbreak in a farm located near Caicara del Orinoco, Venezuela, the blood samples from 48 cows were collected and employed for diagnostic evaluation. Camargo

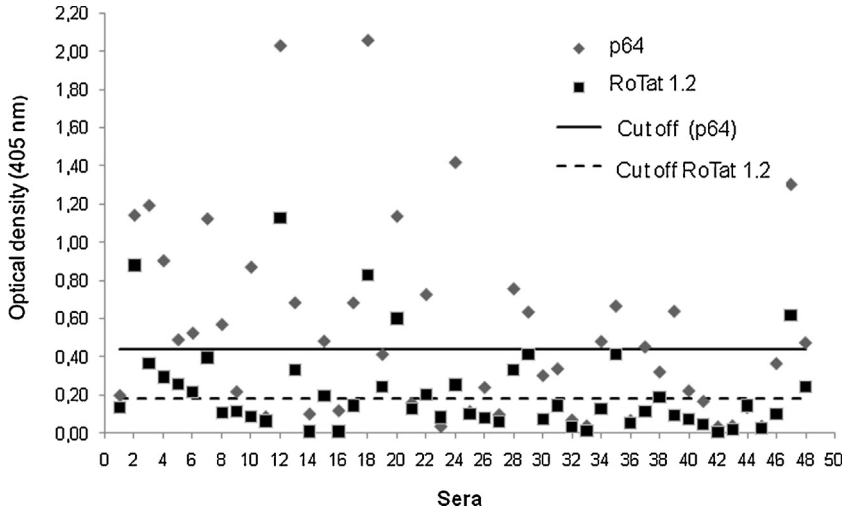

Fig. 3. Comparison of bovine antibody responses against p64 or the RoTat 1.2 VSG by indirect ELISA. p64 (♦) or RoTat 1.2 VSG (■) was used to evaluate the immune response of 48 cows which blood was collected during a bovine trypanosomosis outbreak in a farm located near Caicara del Orinoco, Venezuela. Cut off values for p64 (continuous line) and the RoTat 1.2 VSG (- - -) are shown.

Table 3
Concordance between indirect ELISA using p64 and indirect ELISA using RoTat 1.2 VSG applied on 48 bovine sera obtained during a trypanosomosis outbreak.

	Positive by p64 ELISA [≥0.44]	Negative by p64 ELISA [<0.44]	Total
Positive by RoTat 1.2 ELISA[≥0.18]	18 (37.5%)	2 (4.2%)	20 (41.7%)
Negative by RoTat 1.2 ELISA [<0.18]	6 (12.5%)	22 (45.8%)	28 (58.3%)
Total	24 (50%)	24 (50%)	48 (100%)

Cut off values are shown in square brackets. Percentages are shown in parentheses. p64 ELISA, indirect ELISA using the purified p64 antigen from *T. equiperdum*; RoTat 1.2 ELISA, indirect ELISA using the purified RoTat 1.2 VSG from *T. evansi*.

et al. (2015) have shown that not only p64 but other purified soluble forms of VSGs from various Venezuelan trypanosome isolates are cross-reacting antigens that are recognized by sera from animals infected with either *T. evansi* or *T. vivax*. Since current diagnostic tests for *T. evansi*-caused trypanosomosis are based on antibody detection against RoTat 1.2 VSG, which is a VAT from an Indonesian *T. evansi* isolate, we also investigated here whether RoTat 1.2 VSG exhibited cross-reactivity with *T. vivax* and was capable of being used as a diagnostic tool for non-tsetse transmitted bovine trypanosomosis. As shown in Fig. 3, indirect ELISA was employed to evaluate and compare the responses against p64 and the RoTat 1.2 VSG of the 48 bovine sera mentioned above. Table 3 summarizes the results that were obtained. From these findings, we concluded that similar to Venezuelan VSGs, the Indonesian *T. evansi* RoTat 1.2 VSG also exhibited cross-reactivity with *T. vivax*. However, Venezuelan bovine sera showed higher recognition of p64 than RoTat 1.2 VSG, suggesting that the geographical origin of the parasite stocks might be important.

Thirty four of the 48 bovine blood samples were employed for diagnostic evaluation by MHC and by PCR using specific primers targeting the ITS1 region of the 18S ribosomal gene of *T. evansi* and *T. vivax*. As illustrated in Fig. 4, control amplicon products obtained for *T. evansi*, *T. vivax* and *T. congolense* correspond to DNA fragments of 480 bp, 250 bp and 620 bp, respectively. Most of the infected cows that were positive by PCR contained *T. vivax* (data not shown); however, PCR detected one bovine that was infected with *T. evansi* (data not shown). Fig. 5 summarizes the results obtained with all four tests (MHC, PCR for either *T. evansi* or *T. vivax*, and indirect ELISA against p64 and the RoTat 1.2 VSG). Although PCR appeared to be the best direct method for both positive and negative diagnosis of bovine trypanosomosis, the four tests provided comparable results (Fig. 5).

A substantial agreement between indirect ELISA using p64 and its counterpart using RoTat 1.2 VSG was revealed by the Cohen's

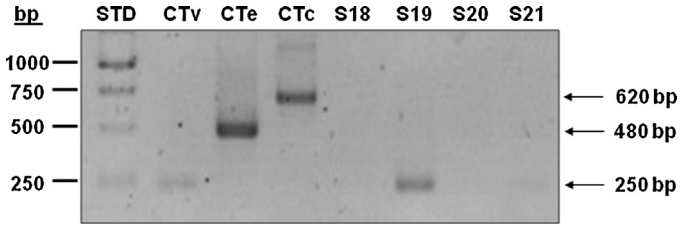

Fig. 4. SYBR® Safe-stained agarose gel electrophoresis of ITS1 PCR products. Control amplicons generated from genomic DNA of the Venezuelan LIEM-176 *T. vivax* isolate (CTv), the Venezuelan TeAp-El Frio01 *T. evansi* isolate (CTe), and the African IL3000*T. congolense* isolate (Ctc) are indicated by arrows. PCR products from blood samples of uninfected bovines (S18, S20 and S21) and a *T. vivax*-infected cow (S19) were also included. STD = 1 kb DNA Ladder.

kappa coefficient ($\kappa = 0.667$). On the contrary, the MHC technique showed no agreement with either of the ELISA tests ($\kappa = -0.184$ and -0.194 when p64 and RoTat 1.2 VSG were used, respectively). PCR showed a fair agreement with the other three methods ($\kappa = 0.081$ for the ELISA with p64, 0.064 for the MHC test, and 0.015 for the ELISA with RoTat 1.2 VSG).

3.4. Agglutination test using p64 coupled to latex microparticles

Initially, we determined the saturation curve of the latex MPP with p64. A saturation point was obtained by using 390 μg of p64 per mg of carboxylate-modified latex MPP (Fig. 6A). Sera from bovines that were identified as positive and negative to trypanosomes by both the MHC assay and an indirect ELISA using p64 as antigen were employed to standardize the agglutination assay. No agglutination was observed when sera from trypanosomosis-infected cows were used in the presence of latex MPP lacking p64 (Fig. 6B). In addition, a homogenous suspension was obtained when p64 covalently bound to latex MPP was mixed

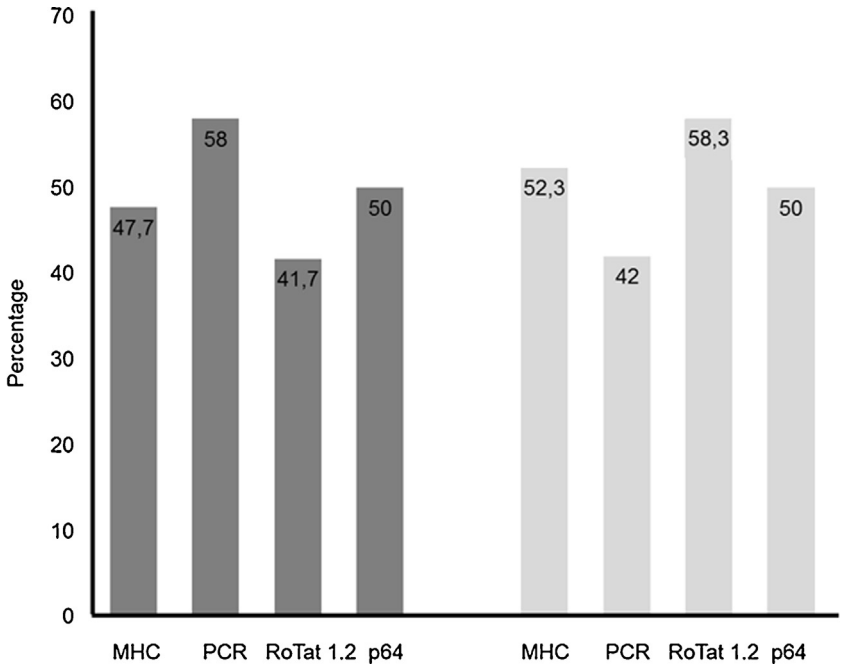

Fig. 5. Comparison of MHC, PCR and indirect ELISA using either p64 or RoTat 1.2 VSG, as methods for the diagnosis of bovine trypanosomosis. Blood samples from 48 cows that were collected during an outbreak in a bovine farm located near Caicara del Orinoco, Venezuela, were employed for trypanosomosis diagnostic evaluation using the MHC technique, PCR and indirect ELISA. Shown is the percentage of bovine samples that were diagnose as positives (left bars, dark grey) or negatives (right bars, light grey) by each of the four tests. p64 = indirect ELISA using p64 as antigen. RoTat 1.2 = indirect ELISA using the RoTat 1.2 VSG as antigen.

and incubated with all of the 20 negative control bovine sera, indicating again that there was no agglutination caused by antigen-antibody complex formation. Fig. 6B illustrates the characteristic negative agglutination response when trypanosome non-infected bovine sera were used (mock serum). In contrast, all 40 positive sera showed the presence of clumps or flocculates, which are typical of a positive agglutination reaction. The formation of these aggregates varied depending on each serum and accordingly, the reactions were qualitatively classified with one cross (+), two crosses (++) or three crosses (+++). Fig. 6B shows examples of the agglutination reactions obtained using sera from *T. vivax*-infected bovines. Summarizing, 42.5%, 30% and 27.5% of the positive sera showed agglutination reactions classified with one cross, two crosses and three crosses, respectively. Since, we employed sera from *T. vivax*-experimentally infected bovines, sera obtained during a bovine trypanosomosis outbreak, and sera from *T. vivax*-infected cows living in a trypanosomosis-endemic and enzootic stable area, the agglutination assay presented here using p64 covalently coupled to latex MPP emerges as a qualitatively suitable and rapid method for the diagnosis of both acute and chronic bovine trypanosomosis.

4. Discussion

We have purified a 64-kDa glycosylated antigen from the Venezuelan TeAp-N/D1 isolate of *T. equiperdum*. Tryptic peptides from p64 were analyzed by liquid chromatography/electrospray ionization-tandem mass spectrometry, and the retrieved sequences yielded only one hit to a putative VSG from *T. brucei* TREU927 (Tb927.4.5460) after searching the NCBInr and the *T. brucei brucei* genome database (Camargo et al., 2015). This result indicated that p64 corresponds to the soluble form of the predominant VSG from *T. equiperdum* TeAp-N/D1. Moreover, p64 appeared to be very sensitive for diagnostic purposes (Uzcanga et al., 2004) and was recognized by anti-*T. vivax* bovine antibodies (Uzcanga et al., 2002, 2004).

A high immunological cross-reactivity between trypanosome species such as *T. evansi* and *T. vivax* has been described (Desquesnes and Tresse, 1996). Accordingly, the OIE has reported that the use of whole cell lysates of *T. evansi* leads to strong cross reactions with *T. vivax*, *T. congolense* and even *Trypanosoma* (Schizotrypanum) *cruzi* (OIE, 2010). Although in vivo outbred murine models of trypanosomosis (CD-1, RjOrl:Swiss mice) have been developed using the IL 1392 strain of *T. vivax* that was originally derived from the Y486 isolate from Africa (Blom-Potar et al., 2010; Chamond et al., 2010; Leeflang et al., 1976), and in vitro non-infective *T. vivax* epimastigote axenic cultures have been reported using the same IL 1392 strain (D'Archivio et al., 2011), the production of *T. vivax* antigens continues to be a limiting factor because most *T. vivax* stocks are restricted to large animals (cows, sheep, goats, pigs, etc.), and possess relatively low level parasitaemias. In contrast, rodents can be readily inoculated in the laboratory with *T. equiperdum* and *T. evansi* to acquire high quantities of parasites to prepare antigens for serological tests. Correspondingly, we have focused on the diagnosis of *T. vivax*-caused animal trypanosomosis by using cross-reacting antigens isolated from Trypanozoon trypanosomes (Camargo et al., 2004; Uzcanga et al., 2002, 2004; Velásquez et al., 2014). In the present study, further evidences were obtained confirming that p64 from *T. equiperdum* TeAp-N/D1 is a cross-reacting antigen suitable to be used as a tool to detect bovine trypanosomosis caused by *T. vivax*.

The time course of experimental infections of bovines with *T. vivax* were evaluated here by measuring whole anti-p64 antibodies and specific anti-p64 IgG and IgM antibodies in animal sera by indirect ELISA. Levels of parasitaemia in *T. vivax*-infected bovines showed characteristic regular oscillations, which represented the emergence, proliferation and immunological elimination of antigenically different populations of trypanosomes. High levels of whole anti-p64 antibodies and specific anti-p64 IgM and IgG isotypes were detected in sera from these bovines during the time course of infection, demonstrating that B-cells did experience immunoglobulin class switching to produce IgG isotypes in *T. vivax*-

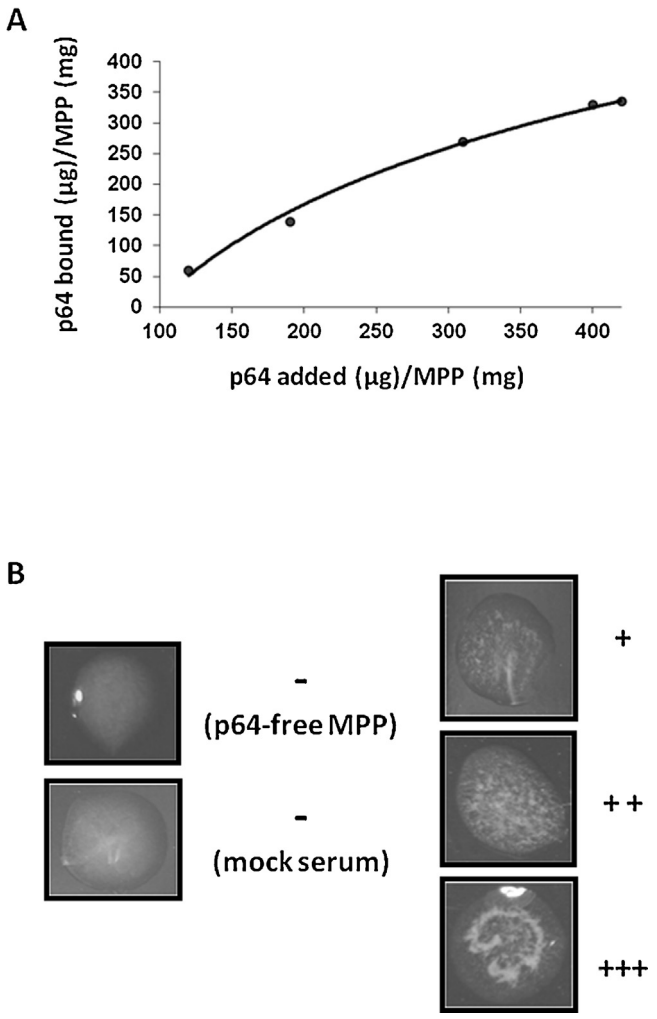

Fig. 6. Agglutination test using p64 coupled to latex microparticles. (A) Saturation curve of carboxylate-modified polystyrene (latex) microparticles (MPP) with p64. (B) Examples of negative agglutination responses (−) that were obtained when sera from trypanosomosis-infected bovines were incubated with p64-free MPP, and when p64-covalently bound MPP was mixed with seronegative (mock) control bovine sera (left). In contrast, formation of clumps indicated a positive agglutination reaction (right). Positive reactions were qualitatively classified with one cross (+), two crosses (++) or three crosses (+++).

infected cows. These results further illustrate that p64 behaves as a cross-reacting antigen between Trypanozoon trypanosomes and *T. vivax* and revealed that p64 has common invariant epitopes that were immunorecognized by bovine sera throughout the whole infection period.

Its diagnostic value was proven by comparing the serological results obtained using p64 with those obtained using either the *T. equiperdum* clarified soluble fraction or the RoTat 1.2 VSG. Moreover, the p64 ELISA results were contrasted to those obtained by PCR targeting the ITS1 region, and by the classical MHC parasitological technique. Our results endorsed the use of p64 in the diagnosis of bovine trypanosomosis generated by non-tsetse transmitted *T. vivax*. We have also shown here that similar to p64 and other soluble forms of VSGs isolated from various Venezuelan animal trypanosomes (Camargo et al., 2015), the RoTat 1.2 VSG exhibited cross-reactivity with *T. vivax* and was capable of being used as a serodiagnostic tool for bovine trypanosomosis in Venezuelan samples. Interestingly, most of the bovine infected sera provided analogous results using either p64 or the RoTat 1.2 VSG, and recognized both antigenic probes; however, some sera recognized exclusively either p64 or RoTat 1.2 VSG. Thus, we recommend the use of a combination of VSG variants for a superior serodiagnosis of non-tsetse transmitted bovine trypanosomosis.

Recombinant VSGs are alternative sources of antigens which appeared to be very useful for the serodiagnosis of animal trypanosomosis. For example, Sengupta et al. (2014) have reported that the RoTat 1.2 VSG expressed in *Escherichia coli* showed 95.6% sensitivity, 98.0% specificity and 0.93Cohen's kappa value when compared with standard antigens, corroborating that the recombinant antigen can be a diagnostic tool to detect carrier animals. Yet, when the diagnostic potential of recombinant LiTat 1.3 and LiTat 1.5 VSGs expressed in the yeast *Pichia pastoris* were compared with their corresponding native antigens by ELISA using sera from *T. brucei gambiense* patients of human african trypanosomiasis (HAT) and non-HAT controls (Rogé et al., 2014), the response obtained for each recombinant with the patient sera was lower than for the corresponding native antigen with the same sera. Although linear epitopes are present in both native and recombinant antigens, native antigens have the advantage that they additionally possess the original three dimensional conformation of the protein and bear all co- and post-translational covalent modifications, which might represent essential epitopes for antigen immunoreactivity.

Accurate diagnosis of trypanosome infection is required for a proper understanding of the epidemiology of the disease, which can then result in the implementation of adequate control strategies (FAO, 1992, 1998). The diagnosis mainly involves examination of clinical signs and laboratory methods. A presumptive field diagnosis is often based on finding an anemic animal with a poor body condition in an epidemic or endemic area. Even then, because of the various clinical manifestations, diagnosis of trypanosomosis cannot be based on these clinical signs only as no pathognomonic clinical sign can confirm the disease (Nantulya, 1990). The presence of the parasite must thus be confirmed to ensure a proper diagnosis. Different techniques are therefore required to be used, such as direct microscopy, concentration techniques, laboratory animal inoculation, detection of anti-trypanosoma antibodies and molecular assays (Moser et al., 1989; Murray et al., 1977; Nantulya, 1990). Each of these techniques presents some strong and weak points (Moti et al., 2014). However, due to the behavior of the vectors, the detection of an infected animal involves testing all animals in the herd (Muzari et al., 2010). In order to determine their sensitivity throughout the course of disease in rabbits experimentally infected with *T. evansi*, Ramírez-Iglesias et al. (2011) compared various diagnostic techniques: two parasitological methods, the MHC test and DME, a serological method using indirect ELISA against the parasite clarified fraction, and PCR. The highest diagnostic register during the course of infection was achieved by the PCR technique (93.8%), followed by indirect ELISA (71.1%), MHC (59%) and DME (13.6%) (Ramírez-Iglesias et al., 2011). In the present paper, Bayesian analysis gives an estimation on the characteristics of four tests to detect trypanosomosis in and endemic area of Venezuela (DME and MHC that detect direct trypanosome infection, and indirect ELISA using either the parasite clarified fraction or the purified p64 antigen that detect exposure). According to the model, a sensitivity value of 71.6% for the p64 indirect ELISA was estimated, which is lower than the sensitivity of 80.8% that was also estimated here for the indirect ELISA using the *T. equiperdum* clarified antigenic fraction, but comparable to the sensitivity of 71.1% reported by Ramírez-Iglesias et al. (2011) for the indirect ELISA using the parasite clarified fraction in *T. evansi* infections. However, when herds of cattle are required to be diagnosed in the field, a purified antigenic protein such as p64 is obviously a more stable diagnostic reagent than a trypanosome clarified fraction, since the latter is a crude extract that contains all parasite soluble proteins, including proteases, glycosidases, etc., which during transportation and/or storage might degrade and hydrolyze essential antigenic epitopes responsible of the serological response. As expected, Bayesian modeling also demonstrates

that antibody detection by indirect ELISA using either the purified p64 or the *T. equiperdum* clarified fraction possesses a higher sensitivity than parasitological tests such as DME and MHC. Given that parasites are directly detected under the microscope, the DME and MHC parasitological tests also had a very high specificity but their sensitivities were low. Previous reports have indicated that these parasitological methods have a detection threshold of around 5000 parasites/ml (Chappuis et al., 2005). Since the parasite number may drop to less than100 parasites/ml, parasitological tests are not generally able of detecting trypanosomes during the low points of parasitaemia that are reached when parasites containing particular VATs are removed by the immune system of the host. Although both indirect ELISA tests detect antibody production (exposure), the specificity for the ELISA using the *T. equiperdum* clarified fraction (83.2%) was lower than the specificity for the p64 ELISA (98.8%), and had a very wide credibility interval. It is anticipated that the use of a crude antigen such as the *T. equiperdum* clarified fraction would have more cross-reactions with many other parasites than a purified antigen such as p64, therefore affecting the specificity of the test. Bayesian analysis also estimated an apparent prevalence of 37.7% for *T. vivax*- caused trypanosomosis when the p64 ELISA was used as diagnostic test. In Venezuela, local and nationwide surveys have been performed to determine trypanosome seroprevalence in bovines, and values ranging from 20% to 80% have been reported (Gonzatti et al., 2014). The most recent study processed a total of 1.675 bovine blood samples for the determination of active infection and 1.572 for serology, which were collected in 49 cattle herds from livestock farms scattered in nine Venezuelan states (Suárez et al., 2009). Suárez et al. (2009) found a general active infection rate of 5.9% by using the MHC method, and an overall seropositivity of 33.1% employing a combination of IIF on smears of blood from *T. vivax*-infected goats and indirect ELISA against a cross-reacting *T. evansi* crude antigenic extract. This value agrees very well with the apparent prevalence value of 37.7% found here with the p64 ELISA test using Bayesian modeling.

Similar to the results reported by Ramírez-Iglesias et al. (2011) while following the course of disease in *T. evansi*-experimentally infected rabbits, our findings also showed that PCR using ITS1 primers appeared to be the best direct method to diagnose bovine trypanosomosis, and revealed that most of the Venezuelan infected cows that were tested contained *T. vivax*. Yet, we found one bovine that was infected with *T. evansi*. Recently, Ramírez-Iglesias et al., 2016 have detected *T. evansi* infections in field populations of cattle by PCR using ITS1 primers. Their results suggested the possible role of bovines as reservoirs in the epidemiology of the disease caused by *T. evansi* in Venezuela (Ramírez-Iglesias et al., 2016). Despite the advantage of using PCR as a diagnostic tool for animal trypanosomosis, it has been scarcely applied to assess the prevalence of this disease on field samples because it is very expensive and time-consuming to be implemented in herds of cattle (Solano et al., 1999). Moreover, it requires technical expertise of high level (Solano et al., 1999). Accordingly, serological tests are probably better methods to assess a large number of animals in a herd at the same time. Serological tests together with the clinical evidence will allow an accurate diagnosis of trypanosomosis and a precise detection of infection in herds. The level of agreement between diagnostic tests was evaluated here using the Cohen's kappa coefficient that showed a close relationship and a significant agreement between the serological tests using p64 and RoTat 1.2 VSG. In contrast, the MHC method showed no agreement with either of the ELISA tests. Additionally, PCR showed a fair agreement with the ELISA using p64, the ELISA using RoTat 1.2 VSG and the MHC test. Overall, none of the tests appeared to be individually sufficient to diagnose bovine trypanosomosis and a combination of several diagnostic tests is strongly recommended.

An agglutination serological assay was designed here using p64 covalently coupled to carboxylate-modified latex MPP, which was proven to be suitable for a fast qualitative detection of anti-trypanosome antibodies. Recently, the N-terminal fragment of the RoTat 1.2 VSG was expressed as a recombinant truncated protein in *P. pastoris* (Rogé et al., 2013), and Rogé et al. (2014) incorporated this recombinant truncated antigen in a latex agglutination test, the rLATEX/*T. evansi*. This test was significantly more specific than the CATT/*T. evansi* assay previously reported (Rogé et al., 2014). Our results revealed that the agglutination test using p64 could be successfully used for diagnosis of *T. vivax* in acute and chronic infections of herds of cows in remote areas where access to indirect ELISA or PCR is limited.

Ethical standards

We declare that all the experiments in this paper were carried out in accordance with the legal and ethical standards of Venezuela.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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