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Comparison of three methods for diagnosis of *Trypanosoma* (*Duttonella*) *vivax* in cattle

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ABSTRACT. Detection of *Trypanosoma vivax* in cattle is based on parasitological, serological and molecular methods. The aim of this study was to compare three different methods for detecting *T. vivax* during an outbreak of trypanosomiasis in southeast Brazil. Blood samples were collected from seventy-two animals to perform micro-hematocrit centrifugation technique (MHCT), indirect fluorescent antibody (IFAT), and polymerase chain reaction (PCR). A total of 84.72% (61/72) of the samples yielded a positive result in at least one test. The PCR test yielded the highest number of positives [73.6% (53/72)], followed by the IFAT [61.1% (44/72)] and MHCT [29.0% (21/72)]. Agreement among the three methods, as expressed by the kappa value, was poor. PCR proved to be the most sensitive of the three methods by demonstrating the highest positivity rate, even in the presence of low parasite levels. This work presents the first study comparing three different detection tests, to *T. vivax*, analyzing a relatively large-scale number of samples.

Key words: Diagnosis; *Trypanosoma vivax.*

INTRODUCTION

Trypanosomiasis represents a threat to the health and productivity of livestock. The disease may cause depression, motor incoordination, transient blindness, anorexia, fever, increased heart and respiratory rates, weight loss, anemia, and infertility in infected animals (Betancur Hurtado et al., 2016). The causative agents are various species of protozoan parasites belonging to the genus Trypanosoma, among which T. (Duttonella) vivax Ziemann (1905) plays an important role as an etiological agent of bovine trypanosomiasis in many countries (Gonzales et al., 2003; Delafosse et al., 2006). This protozoan may be transmitted by the biological vector, tsetse flies (*Glossina* spp.), or mechanically, by other blood-sucking flies (Batista et al., 2012). Clinical signs along with the use of accurate diagnostic tools are required to confirm the presence of *T. vivax*. There are several methods for detection of animal trypanosomiasis, including parasitological, immunological and molecular methods, with varying advantages and disadvantages depending on the stage of disease at which they are applied (Osório et al, 2008; Ramírez-Iglesias et al., 2011; Maganga et al., 2017). In view of the need to develop efficient control strategies and considering the paucity of published information on *T. vivax* infection in many countries, the aim of this study was to evaluate the efficacies of three different diagnostic tests for detecting *T. vivax* infection in cattle.

MATERIAL AND METHODS

A total of 144 blood samples were collected from 72 cows in 8 farms during an outbreak in Minas Gerais, Brazil. Each animal was bled from the tail vein using a vacutainer tube, and the blood samples were kept on ice during transportation to the laboratory. Half of the samples were collected into tubes containing EDTA and sent to the laboratory for direct parasitological and molecular testing. The other 72 samples were collected into tubes without anticoagulant for serologic testing. Plasma and blood were separated and stored in 1.5 ml microtubes at

−20°C until processing for serological and molecular tests, respectively. All animal procedures were approved by the Animal Care and Use Committee of the Universidade Federal de Minas Gerais (Protocol 143/08).

For each animal, three different methods for *T. vivax* detection were performed: Micro-hematocrit centrifugation technique (MHCT), indirect fluorescent antibody test (IFAT), and polymerase chain reaction (PCR). For the MHCT, micro-haematocrit capillary tubes were filled with the EDTA-blood samples, sealed and centrifuged at 1200 × g for 5 min. The buffy coat, in which motile trypanosomes can be viewed, between the leukocyte layer and the plasma was examined under the microscope (Woo, 1970). When the MHCT was positive, the capillary tube was cut and a buffy coat smear was prepared (Murray et al., 1977). The smears were stained with Giemsa, and trypanosomes were identified based on morphological and biometrical data (Hoare, 1972).

DNA was extracted from 300 µL of each blood sample using WizardTM Genomic DNA Purification Kit (Promega, Madison, Wisconsin, United States). The PCR test was a semi-nested assay. The first PCR reaction produced a fragment of 500 bp and was performed using primers DTO154 (5' GGG CCA ATG CGG CTC GTG CTG G 3') and DTO155 (5' TTA GAA TTC CCA GGA GTT CTT GAT GAT CCA GTA 3'). The semi-nested PCR was carried out using the primers TviCatL (5' GCC ATC GCC AAG TAC CTC GCC GA 3') and DTO155, producing a fragment of 177 bp corresponding to a region exclusively from *T. vivax* genomic DNA (partial fragment of the cathepsin L-like gene), as described previously (Cortez et al., 2009). The reactions were performed in a total volume of 10 µL, containing 20-100 ng of genomic DNA, 0.2 µL of each primer (10 µM) and 5 µL of GoTaqTMGreen Master Mix (Promega, Madison, Wisconsin, United States). The reactions were performed for 30 cycles at 92°C (1 min), 56°C (1 min) and 72°C (2 min) followed by a final extension of 8 min at 72°C. To confirm the PCR results, amplicons from three positive samples were sequenced. Following the first reaction (primers DTO154 and DTO155), a second reaction was performed using the primers CatTvi1 (5' GTG GTG GCC TGA TGG ACA A 3') and CatTvi2 (5' TAC GGC GGC TTG CTG CTG 3') which produced a 341 bp fragment including the 177 bp *T. vivax*-specific sequence, according to the protocol described above. The resulting amplicons were sequenced directly using the same primers (CatTvi1 and CatTvi2) according to the method described by Sanger et al. (1977).

To prepare the antigen used in IFAT, a splenectomized two-month-old goat was inoculated subcutaneously with the cryopreserved stabilate of *T. vivax*. When parasitaemia reached a value of 2 × 107 trypanosomes/ml, 100 ml of blood was collected from the goat jugular vein in the presence of EDTA-Na2 (0.15%). The blood was mixed with an equal amount of Percoll (Sigma-Aldrich, St. Louis, Missouri, United States) containing 8.55% sucrose and 2.0% glucose (density 1.130 ml−1), and the pH was adjusted to 7.4 with HEPES and centrifuged at 17,500 × g for 20 min at 4 °C. The parasites were recovered and resuspended in PBSG (40 mM NaH2PO4, pH 7.5 and 150 mM NaCl with 1% glucose) in the proportion of 1:3. The trypanosomes were analyzed by chromatography on a diethylaminoethyl (DEAE) cellulose column and fixed onto the slide glass.

The presence of anti-*T. vivax* IgG antibodies was detected in plasma by an IFAT using rabbit anti-bovine-IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, Missouri, United States) and acetone-fixed *T. vivax* trypomastigotes (Katende et al., 1987). A positive serum sample was obtained from a calf previously experimentally infected with *T. vivax*. Only samples reactive at a dilution equal to or greater than 1:80 were considered positive.

The percentage of positive samples by each test was calculated and the agreement among the tests was based on the analysis of the kappa statistics, using an online kappa calculator (http://www.lee.dante.br/pesquisa/kappa/). The values of kappa range from -1 to +1, with -1 indicating perfect disagreement and +1 indicating perfect agreement between the raters. The results were used to determinate the strength of agreement, which ranged from "poor" to "almost perfect," according to Landis and Koch (1977). In addition, the proportions of positive and negative agreement (ppos and pneg, respectively) were calculated.

RESULTS AND DISCUSSION

A total of 84.7% (61/72) of the samples were positive by at least one of the three tests. This high positivity rate can be explained by the fact that the blood samples originated from an outbreak region. However, when the results of each method were compared, there were dramatic differences among the numbers of positive animals (Figure 1a). The highest prevalence was observed using the PCR test, which resulted in a positivity rate of 73.6% (53/72), confirmed by sequencing. Figure 2 shows a representative agarose gel image with

the expected amplicon of ~ 177 bp as the reaction product. This high detection rate observed corroborates the high sensitivity of this PCR technique, which can detect DNA from ~ 2 parasites/mL3.

The parasitological test showed a very low sensitivity, with trypanosomes being detected in only 21 of the blood samples (29%). The effectiveness of parasitological methods as diagnostic tools is limited by low sensitivity, which prevents them from identifying animals with chronic infections and low levels of parasitemia, as demonstrated by Desquesnes and Tresse (1996), who evaluated the sensitivity of MHCT for the detection of *T. vivax* and found generally negative results below 60 parasites/mL. Agreement between MHCT and PCR results in the present study occurred only in samples with parasite loads ranging from 300 to 700 parasites/mL.

The PCR assay was able to detect trypanosome DNA in 35 samples that were negative by MHCT (Figure 1B). However, *T. vivax* was detected in 3 samples negative by PCR when tested by MHCT, indicating that the PCR also had some false-negative results. Desquesnes and Dávila (2002) distinguished two stages of the infection process; an early phase, when parasitological and PCR techniques show very similar sensitivities (80.0%), and a chronic phase, when parasitological assessment results in very low sensitivity (<10.0%) and PCR is two to three times more sensitive. However, the chronic phase of the disease is characterized by low levels of parasitemia, thus limiting the effectiveness of classical parasitological diagnostic tools (Ramírez-Iglesias et al., 2011).

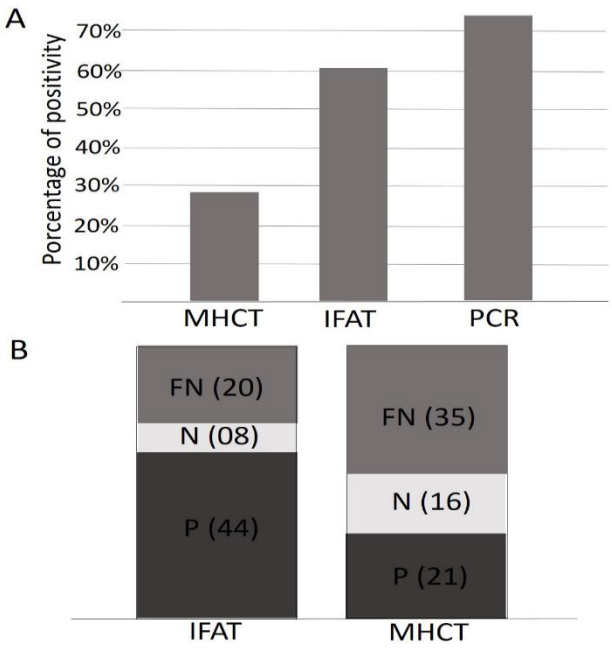

Figure 1. (A) Percentage of detection (PD) by each diagnostic method evaluated. Micro-hematocrit centrifugation technique (MHCT), 29.17% (21/72); Indirect immunofluorescence test (IFAT), 61.11% (44/72); Polymerase chain reaction (PCR), 73.60% (53/72). (B) Proportions of the numbers of positive, negative and false-negative samples by IFAT and MHCT using PCR as the gold standard. P: positive samples; N: negative samples; FN: false-negative samples (negatively diagnosed by the first assay but positively diagnosed by PCR).

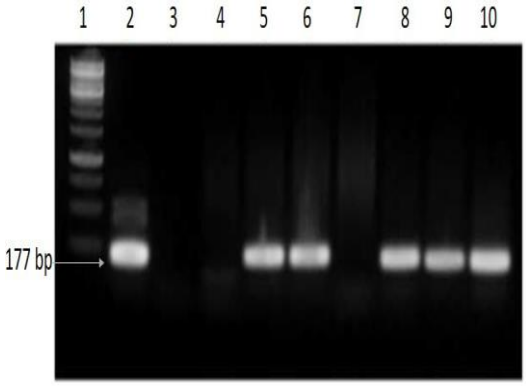

Figure 2. Representative PCR results from the screening of the 72 analyzed samples, showing positive and negative results. (1) Molecular weight marker (1 kb) DNA Ladder (Invitrogen); (2) known positive sample used as a control; (3) "blank sample"; (5, 6, 8, 9 and 10) positive samples; (4 and 7) negative samples. The image shows an agarose gel 1.5%, stained with GelRedTM 458 (Biotium, USA).

Using IFAT as a serological test, antibodies against trypanosoma antigens were found in 61.1% of the samples (44/72). The frequency of antibody-positive animals was more than twice the number of positive samples obtained by direct observation (MHCT). Cuglovici et al. (2010) also found a great difference between the prevalence obtained by the parasitological technique (7.0%) and that observed using antibodies against *T. vivax* (32.0%). Eleven samples were positive in the IFAT but negative by PCR. Indeed, the detection of antibodies is not always indicative of active infection because antibody persistence can occur over the course of several months following curative treatment (Delafosse et al., 2006). A total of 20 IFAT-negative samples were positive by PCR (Figure 1B). This can be explained by the fact that parasitemia precedes antibody development, thus yielding negative IFAT results in the early stages of infection.

The analysis of agreement revealed low kappa values (Table 1), corresponding to poor agreement among the methods used, even though the coefficients were all statistically significant. According to the kappa value, the strongest agreement was between the parasitological and PCR assays (k = 0.118), followed by the parasitological and serological assays (k = 0.110) and PCR and serological assays (k = 0.038). Gonzales et al., (2003) also found a more significant agreement between MHCT and PCR (k = 0.389) when comparing the PCR technique to an immunological test for detection of *T. vivax* (k = 0.030).

Table 1. Agreement among the three methods used for diagnosing *Trypanosoma vivax*.

	Concordant results		Kappa values	*P* value	Confidence Interval (95%)	p_{pos}	p_{neg}
	Positives	Negatives					
MHCT × PCR	18	16	0.118	0.135	-0.037-0.273	0.48	0.45
MHCT × IFAT	15	22	0.110	0.249	-0.077-0.298	0.46	0.56
PCR × IFAT	33	8	0.038	0.737	-0.184-0.260	0.68	0.34

Conversely, the observation of the number of concordant results for each pair of methods compared in this study, revealed a paradox. Considering only the number of concordant (positive and negative results), PCR test and IFAT showed the lowest kappa coefficient. This finding may be explained in this case by the discrepancy between ppos and pneg. The calculation of kappa involves a crucial component that is a product of two increments, one of which is (ppos - pneg) or (a – d) and represents the disparity in agreement between the

negative and positive ratings. If disparate ppos and pneg values are directly displayed, the discrepancy is immediately evident, and its existence can be recognized without the need for recognition by an unexpectedly low kappa value (Cicchetti and Feinstein, 1990). For this reason, in addition to each kappa value, the respective values of ppos and pneg are presented for a better and clearer understanding of the results (Table 1).

In general, MHCT has only shown high sensitivity in cases with high parasitemia levels (Desquesnes and Dávila, 2002). However, Mattioli and Wilson (1996) reported that high levels of antibodies are efficient for maintaining low levels of parasitemia. Therefore, the seroconversion of the animal and the increasing levels of anti-*T. vivax* antibodies contribute to the decreased numbers of parasites in the blood, and consequently, reduce the efficacy of the parasitological method at that stage. This may explain the poor agreement between the results obtained by MHCT and IFAT.

The samples used in this study were collected from animals infected by *T. vivax* during afield outbreak, when MHCT sensitivity is typically low due to animals being in the prepatent period or in a non-parasitemic chronic phase; however, the molecular technique was able to detect the presence of the parasite. The number of samples with parasite numbers below the detection level of the parasitological method contributed to the high number of false negatives, which totalled 35 samples using this technique. These samples were subsequently identified as positive by PCR. This finding also explains the significant levels of disagreement between the molecular and parasitological tests, which resulted in a low kappa value.

The results of the present study provide evidence of the advantages of PCR for detection of *T. vivax*. Despite the fact that in field situations, there is a high probability of animals is coinfected with different hemoparasites, including other Trypanosomatidae, the molecular method was able to detect the parasite with accuracy according to the sequencing results.

This work presents results concerning the methods of detection of an important cattle parasite. It was demonstrated that the PCR technique was more sensitive than the parasite detection method and the agreement among the three methods was generally poor, indicating that each technique has advantages and disadvantages, depending upon the phase of the disease. This information is crucial for the design of strategies aiming the control of this disease.

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