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Original Article

Comparison of conventional and molecular techniques for *Trypanosoma vivax* diagnosis in experimentally infected cattle

Comparação de técnicas convencionais e moleculares para diagnóstico em bovinos experimentalmente infectados por *Trypanosoma vivax*

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Abstract

Livestock infections by *Trypanosoma vivax* have been occurring with increasing frequency, mainly due to the presence of animals with subclinical infections and without apparent parasitaemia, making diagnosis challenging. The aim of the present study was to evaluate several techniques used for *T. vivax* diagnosis in order to assess the best way of using them during the course of the disease. Molecular methods demonstrated higher rates of detection than parasitological methods, detecting 33 of the 54 (61.1%) known positive samples, while the hematocrit centrifugation technique (best parasitological test) detected only 44.4%. The serological methods, IFAT and ELISA, detected seropositivity in 51 of the 54 (94.4%) and 49 of the 54 (90.7%) known positive samples, respectively. Despite being highly sensitive, the latter only demonstrates exposure to the infectious agent and does not indicate whether the infection is active. The present study was the first to use the qPCR for a South American isolate, improving disease detection and quantification. Furthermore, the analyses revealed that the patent phase of the disease may extend up to 42 days, longer than previously reported. The combination of several diagnostic techniques can lower the frequency of false negative results and contributes toward better disease control.

Keywords: PCR, qPCR, serology, trypanosomosis, diagnosis.

Resumo

Infecções por *Trypanosoma vivax* têm ocorrido com frequência crescente em animais de produção, principalmente pela aquisição de animais com infecções subclínicas e sem aparente parasitemia, o que dificulta o diagnóstico. O objetivo do presente estudo foi avaliar várias técnicas empregadas para o diagnóstico de *T. vivax*, a fim de verificar a melhor maneira de utilizá-las durante o curso da doença. Os métodos moleculares demonstraram maiores taxas de detecção que os métodos parasitológicos, detectando 33 das 54 (61,1%) amostras sabidamente positivas, enquanto a técnica de hemoconcentração (melhor teste parasitológico) detectou apenas 44,4%. Os métodos sorológicos, RIFI e ELISA, detectaram soropositividade em 51 das 54 (94,4%) e 49 das 54 (90,7%) amostras sabidamente positivas, respectivamente. Apesar de serem altamente sensíveis, estes testes apenas demonstram a exposição ao agente infeccioso, e não indicam se a infecção permanece ativa. O presente estudo foi o primeiro a utilizar a qPCR para um isolado sul-americano, melhorando sua detecção e quantificação. Além disso, as análises revelaram que a fase patente da doença pode se estender por até 42 dias após a infecção, sendo maior que anteriormente relatado. A combinação de várias técnicas de diagnóstico pode evitar a frequência de resultados falso-negativos e contribuir para um melhor controle da doença.

Palavras-chave: PCR, qPCR, sorologia, tripanossomose, diagnóstico.

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Introduction

Trypanosomosis is a widely distributed parasite-associated disease that affects humans and animals. *Trypanosoma vivax* cause significant economic losses in livestock, being responsible for major losses in sub-Saharan Africa and Central and South America (DÁVILA & SILVA, 2000). Infections by *T. vivax* in livestock have been occurring with increasing frequency in tropical and subtropical regions (DÁVILA & SILVA, 2000; GIORDANI et al., 2016). In cattle, *T. vivax* diagnosis is difficult, since the parasite triggers non-specific symptoms such as fever, anorexia, weight loss, reduced milk production, abortions, and neurological signs (BATISTA et al., 2007; CADIOLI et al., 2012). Moreover, there are fluctuations in parasitaemia and apparently aparasitemic intervals (DESQUESNES, 2004; CADIOLI et al., 2015; FIDELIS et al., 2016).

Direct parasitological methods present poor sensitivity during low parasitaemia or aparasitemic periods, which are common in chronic infection (DESQUESNES, 2004; CADIOLI et al., 2015). Serological tests, including indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and immuno-chromatographic rapid diagnostic test (RDT), are the tools of choice for herd screening but do not indicate if the infection is active or if the animal has responded to treatment (CADIOLI et al., 2012, 2015; BOULANGÉ et al., 2017). On the other hand, molecular methods such as conventional polymerase chain reaction (PCR) and real-time PCR (qPCR) have the potential to be excellent diagnostic tools; however, these techniques are subject to false negative results when the parasitaemia is below the detection level (CADIOLI et al., 2015; KATO et al., 2016).

For trypanosomiasis control, integrated approaches such as vector control, improved general health and immunocompetence of affected herds, and the use of more sensitive and rapid diagnostic tools are necessary (GIORDANI et al., 2016). Thus, the present study aimed to evaluate the use of several techniques for *T. vivax* diagnosis during the course of the disease in order to assess the best way of use them.

Materials and Methods

Experimental infection and sample collection

The present experiment was approved by the Animal Ethics Committee of the São Paulo State University (Unesp) School of Agricultural and Veterinarian Sciences under the process number 13219/15. Three Girolando cows aged six to seven years were experimentally infected via the intravenous route with 2.0×10^7 trypomastigotes of *T. vivax*, "Lins" isolate (CADIOLI et al., 2012; GARCIA et al., 2014), being this a complementary work to the study developed by Fidelis et al. (2016). Blood sampling of each animal occurred seven day before inoculation (–7 DAI), on the day of inoculation (0 DAI), the day after inoculation (1 DAI) and then weekly till 119 days after infection (DAI). At each bleed, 3 mL of whole blood was obtained by jugular venipuncture and collected into a vacutainer tube containing 10% K2-EDTA, and 10 mL was collected into an anticoagulant-free vacutainer tube (B.D. – Juiz de Fora – MG). Blood and serum samples were split into triplicates and stored at –80 °C until required for analyses. Samples from the 1 DAI until 119 DAI were considered as positives.

Parasitological methods

The EDTA-treated blood was used to assess the presence of the parasite and quantify the level of parasitaemia on the day of sampling. Parasites were detected by the hematocrit centrifugation technique as described by Woo (1970), and blood smears were stained with the May-Grunwald-Giemsa. For parasite quantification, the thick-drop counting method described by Brener (1961) was performed using 5 µL of whole blood placed on a microscope slide under a 22 mm^2 coverslip. Trypomastigote forms were counted in 50 microscopic fields under a 40X objective. The parasite count was multiplied by the microscope correction factor, and the result expressed in parasites per mL blood (BRENER, 1961).

Serological methods

In order to obtain antigen for use in the IFAT and ELISA methods, trypomastigotes were purified from the whole blood of a goat experimentally infected with *T. vivax*, "Lins" isolate (CADIOLI et al., 2012; GARCIA et al., 2014). Purification was performed as described by González et al. (2005).

IFAT was conducted as described by Aquino et al. (1999) with minor modifications. Teflon printed diagnostic slides (Perfecta®, São Paulo, Brazil) previously coated with purified *T. vivax* trypomastigotes were thawed at room temperature for 10 min and to each well, successive dilutions of each test serum sample were added, starting at 1:80 and ending at 1:1280. Samples that were reactive at the 1:80 dilution were considered seropositive.

The ELISA was carried out as described by Aquino et al. (1999), with minor modifications as described below. Each microplate well (Nunc MaxiSorp®) was coated with 100 µL of the soluble antigen at a concentration of 400 ng/mL. Sera were tested in duplicate, and positive and negative controls were tested in quadruplicate. All samples and controls were diluted 1:50 in phosphate-buffered saline with Tween-20 ([PBST]; 130 mM NaCl, 2.7 mM KCl, 5.6 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.92 mM NaH_2PO_4 and 0.05% Tween 20). The reaction was read by a microplate reader (MRX TC Plus, Dynex Technology, USA) at 405 nm. The blank well did not contain serum. The mean absorbance and standard deviation for the positive and negative control serum samples were 1.121 ± 0.081 and 0.234 ± 0.034, respectively. The cut-off point was calculated as 0.335 as described by Madruga et al. (2006).

Molecular methods

The DNA extraction was performed with the DNeasy Blood & Tissue Kit® (Qiagen, Germantown, MD, USA) according to the manufacturer's recommendations, using an aliquot of 200 µL of blood. The extracted DNA was stored at –20 °C until required.

PCR was performed using a set of primers based on the DNA sequence of the *T. vivax CatL* gene as described by Cortez et al. (2009), in a final volume of 50 µL containing

a specific PCR mixture: (1) 20–100 ng of genomic DNA (except the negative control); (2) 100 pmol of DTO 155 (5'-TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA-3') and TviCatL1 (5'- GCCATCGCCAAGTACCTCGCCGA-3') primers; (3) 200 μM of each dNTP; (4) 20 mM of Tris–HCl (pH 8.4); (5) 50 mM KCl; (6) 1.5 mM of $MgCl_2$; (7) 7.5% (v/v) dimethyl sulfoxide; (8) 0.1 mg/mL of bovine serum albumin; and (9) 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Reactions were conducted in a thermocycler T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) under specific conditions: (1) initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min; (2) annealing at 65 °C for 1 min and extension at 72 °C for 1 min; and (3) a final extension at 72 °C for 10 min. A positive control template, genomic DNA from *T. vivax*, isolate "Lins" (GARCIA et al., 2014), was included also. The PCR products were separated on 2% agarose gel containing ethidium bromide. The gel image was obtained using the ChemiDoc™ MP imaging system (Bio-Rad). DNA size standards (GeneRuler 50 bp DNA Ladder; Thermo Fisher Scientific; Waltham, MA, USA) were incorporated in the gel. Relative quantification of the band intensities was performed using the Image Lab™ Software 5.2.1 (Bio-Rad). The intensities of the PCR products were compared to the intensity of positive control product of each gel run.

TaqMan qPCR was performed as described by Silbermayr et al. (2013). Primers for concurrent detection of the ITS1 regions of *T. congolense, T. brucei* and *T. vivax* and for the bovine *toll-like-receptor* 8 (*TLR*-8) (endogenous gene) were initially used with the addition of probes labelled with FAM fluorophore and BHQ2 quencher for *T. vivax* detection and HEX fluorophore and BHQ2 quencher for *TLR*-8 detection. Reactions were performed using 5 μL of genomic DNA (except for the negative control), 200 nM of Tryps_KS-for (5'-CGTGTCGCGATGGATGACTT-3'), Tryps_KS-rev (5'- CAAACGGCGCATGGGAG-3'), TLR8-for (5'- TGTTTAGAGGAAAGGGATTGGG-3') and TLR8-rev (5'-TTGGTTGATGCTCTGCATGAG-3') primers, 160 nM of *T. vivax* (FAM–ATGACCTGCAGAACCACTCGATTACCCAGT–BHQ2) probe, 120 nM of *TLR*-8 (HEX–CCCGGGTCTAGCCATCATCGACAA–BHQ2) probe, buffer 2X (6 nM of $MgCl_2$, 0.8 mM of dNTPs and 1 U of Taq DNA polymerase, GoTaq Hot Start Polymerase (Promega, Madison, USA)). The final volume was 25 μL. qPCR amplifications were conducted in low-profile 96-well unskirted PCR plates (Bio-Rad) using a CFX96 thermal cycler (Bio-Rad) under specific conditions: (1) initial denaturation at 95 °C for 10 min; (2) 45 cycles at 95 °C for 30 s and 61 °C for 1 min; and (3) termination at 72 °C for 1 min. All samples were processed in duplicate.

The sensitivity of the qPCR assay was tested with gBlock® Gene fragments (Integrated DNA Technologies®, Coralville, IO, USA) containing the target sequences for amplification of *T. vivax* ITS1 region. Serial dilutions were made in order to construct patterns with different concentrations of gBlock® containing the target sequence (2.0×10^7 to 2.0 copies/μL). The copy number was determined according to the formula (X g/μL DNA/ [gBlock® size (bp) × 660]) × 6.022×10^{23} × copies of gBlock®/μL). The amplification efficiency (E) was calculated according to the slope of the standard curve of each run according to the following formula ($E = 10^{-1/slope}$).

Statistical analysis

The Kappa concordance test was performed between the different techniques used. In addition, two Spearman's correlation tests were performed. The first was between the values obtained from the parasitaemia estimated by the thick-drop count and those determined by qPCR, and the second correlation was determined for the relationship between the qPCR values and those obtained by the relative intensity of the PCR products.

Results

The results are presented in Table 1 and Figure 1 and show the presence of trypomastigotes from the second post-infection collection (7 DAI) as verified by the three parasitological methods. Among the methods used for direct parasite detection, the hematocrit centrifugation technique had the highest detection capability, detecting 24 of 54 known positive samples (44.4%). However, the thick-drop and stained blood smear techniques showed similar detection capabilities, detecting 17 of 54 known positive samples (31.5%) (Table 1).

The IFAT and ELISA techniques performed comparably with the IFAT being slightly more sensitive, detecting seropositivity in 51 of the 54 known positive samples (94.4%). From 7 DAI, all animals showed reactivity at the 1/80 serum dilution and after 21 DAI achieved seropositivity at the 1/1280 dilution, maintaining this level (or higher) until the end of the experimental period. The exception was the E1 animal, whose titre dropped to 160 at 119 DAI. The ELISA test detected seropositivity in 49 of the 54 known positive samples (90.7%). At 7 DAI only one animal (E1) was seropositive, but from 14 DAI, all samples were seropositive and remained so throughout the experiment (Table 1).

Regarding the molecular evaluation, all samples were positive for the host endogenous gene *TLR-8*, indicating that extractions were efficient, and showing that the results obtained by both PCR and qPCR are reliable. The mean and intervals for efficiency, R^2, slope, and y-intercept of qPCR reactions were 93.6%, (90.1–97.1), 0.988 (0.970–0.999), –3.487 (–3.586–(–)3.392) and 39.758 (38.583–41.764), respectively. All duplicates presented a maximum variation of 0.5 Cq. Both techniques detected the *T. vivax* DNA in 33 of the 54 known positive samples (61.1%) with the first detection on the first day after infection (1 DAI) (Table 1 and Figure 1).

The highest values of parasitaemia, detected by the thick-drop technique, occurred on 14 DAI with 6.82×10^6, 1.62×10^7 and 3.99×10^6 parasites/mL of blood for E1, E2 and E3 cows, respectively. The quantification by qPCR also revealed that the highest parasite values occurred on 14 DAI, showing 5.03×10^7, 2.70×10^8 and 8.53×10^7 copies of the target region/mL of blood, for E1, E2 and E3 animals, respectively (Table 1 and Figure 1). Fluctuations in parasitaemia were observed during the experimental period both by qPCR and thick-drop techniques, highlighting a patent phase, in which circulating parasites were seen throughout the entire period, and subpatent phase, in which a low parasitaemia was detected followed by aparasitemic periods. The parasitemic curves quantified by the qPCR technique are shown in Figure 1,

Table 1. Results for *T. vivax* detection by different methodologies in three experimentally infected cows (E1, E2 and E3).

DAI	Direct Parasitological Methods									Molecular Methods						Serological Methods					
	Hematocrit centrifugation			Stained blood smear			Thick-drop (parasites/mL)			qPCR (copies/mL)			PCR (Relative intensity)			IFAT			ELISA (OD*)		
	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3
-7	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	0.237	0.253	0.278
0	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	0.238	0.311	0.287
1	-	-	-	-	-	-	0	0	0	26.8	29.1	49.8	+ (0.13)	+ (0.03)	+ (0.18)	-	-	-	0.332	0.305	0.314
7	+	+	+	+	+	+	8100	8100	2502900	12800	24800	75000000	+ (0.50)	+ (0.36)	+ (2.14)	1:80	1:80	1:80	0.490	0.331	0.303
14	+	+	+	+	+	+	6820200	16248600	3985200	50300000	270000000	85300000	+ (0.93)	+ (1.04)	+ (0.83)	1:640	1:1280	1:1280	0.415	0.627	0.490
21	+	+	+	+	+	+	8100	32400	8100	40600	447	16700	+ (0.50)	+ (0.56)	+ (1.01)	1:1280	1:1280	1:1280	0.358	0.616	0.593
28	+	+	+	-	-	-	64800	0	0	4310	14400000	108000000	+ (0.67)	+ (1.26)	+ (2.11)	1:1280	1:1280	1:1280	0.492	0.604	0.561
35	+	+	+	+	+	+	8100	145800	113400	76	19900000	2030000	+ (0.32)	+ (0.97)	+ (1.43)	1:1280	1:1280	1:1280	0.509	0.513	0.649
42	+	+	+	-	-	-	0	0	0	32.3	16.9	23.1	+ (0.17)	+ (0.03)	+ (0.07)	1:1280	1:1280	1:1280	0.450	0.869	0.719
49	+	-	-	-	-	-	0	0	0	19.2	65.3	0	+ (0.13)	+ (0.04)	+ (0.10)	1:1280	1:1280	1:1280	0.374	0.906	0.834
56	-	+	-	+	+	-	0	81000	0	0	127000	232	+ (0.05)	+ (1.54)	+ (0.01)	1:1280	1:1280	1:1280	0.408	0.874	0.763
63	+	-	+	-	-	+	0	0	8100	734	55.9	22.3	+ (0.70)	+ (0.02)	-	1:1280	1:1280	1:1280	1.117	1.046	0.833
70	-	-	+	-	-	+	0	0	113400	0	0	347100	-	-	+ (0.88)	1:1280	1:1280	1:1280	0.785	1.051	0.687
77	-	-	-	-	-	-	0	0	0	65.5	0	0	+ (0.09)	-	-	1:1280	1:1280	1:1280	0.508	1.242	0.602
84	-	-	-	-	-	-	0	0	0	13700	0	0	+ (0.40)	-	-	1:1280	1:1280	1:1280	0.379	1.007	0.592
91	-	-	+	-	-	+	0	0	1012500	0	0	45700000	-	-	+ (0.90)	1:1280	1:1280	1:1280	0.343	1.029	0.862
98	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:1280	1:1280	1:1280	0.368	1.246	0.913
105	-	-	-	-	-	-	0	0	0	0	0	1000	-	-	-	1:1280	1:1280	1:1280	0.486	1.185	0.834
112	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:1280	1:1280	1:1280	0.453	0.953	0.733
119	-	-	-	-	-	-	0	0	0	0	0	0	-	-	-	1:160	1:1280	1:1280	0.367	0.778	0.676

-, Negative; +, Positive; *OD (optical density) > 0.335 = Positive.

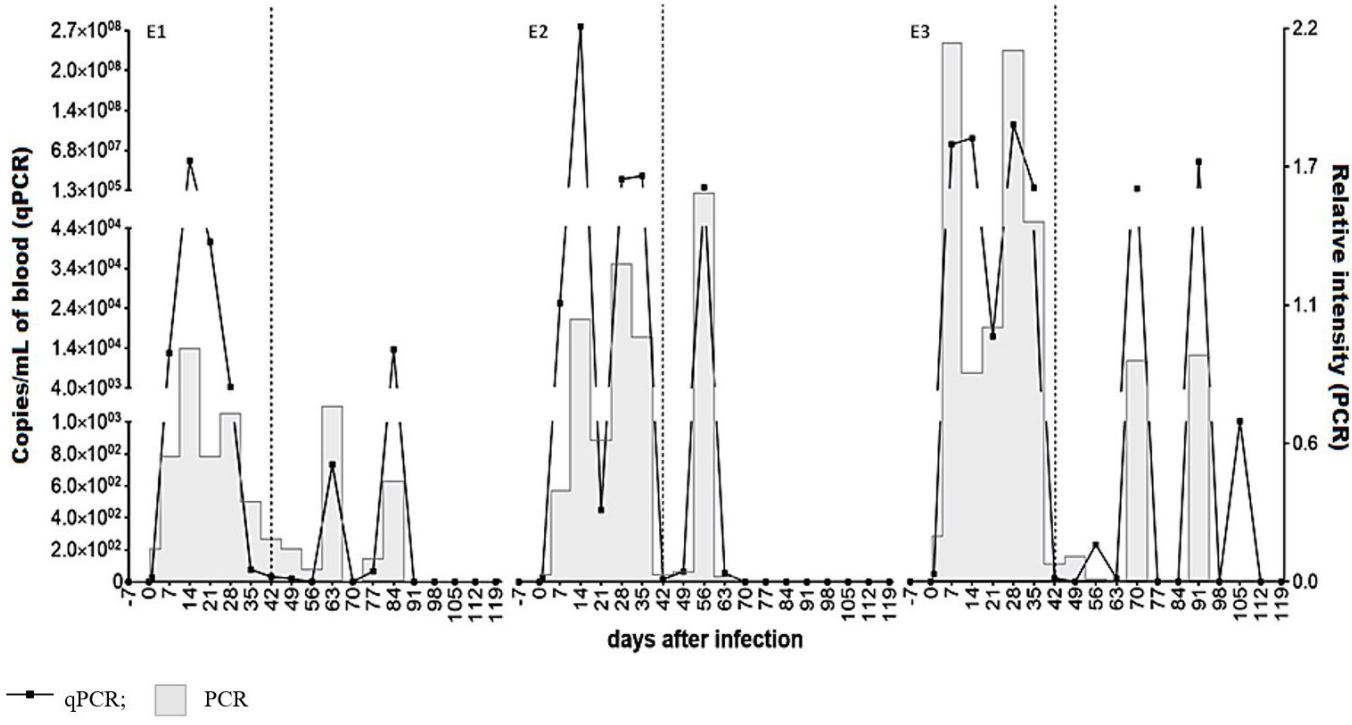

Figure 1. Parasitemic curves quantified by qPCR and relative intensity of PCR of three individual cows (E1, E2 and E3) experimentally infected with *T. vivax*.

Table 2. Results for Kappa concordance test between the several techniques employed for *T. vivax* diagnosis.

	Hematocrit centrifugation	Blood smear	Thick-drop	IFAT	ELISA	PCR	qPCR
hematocrit centrifugation							
blood smear	0.67						
thick-drop	0.74	0.92					
IFAT	0.21	0.13	0.13				
ELISA	0.14	0.06	0.06	0.88			
PCR	0.64	0.42	0.42	0.14	0.07		
qPCR	0.71	0.42	0.49	0.14	0.07	0.87	

while the parasitemic curves quantified by thick-drop technique were reported in Fidelis et al. (2016).

Kappa agreement results are presented in Table 2. Both correlation analyses were shown to be positive according to Spearman r = 0.8757 (P <0.0001) for the two quantitative techniques, thick-drop technique and qPCR, and Spearman r = 0.9151 (P <0.0001) for the qPCR quantification and PCR relative intensity.

Discussion

South American isolates of *T. vivax* do present some genetic differences despite being phylogenetically related to African isolates (GARCIA et al., 2014). The present study was the first to use the qPCR protocol, developed for African isolates (SILBERMAYR et al., 2013), for one South American isolate, detecting the *T. vivax* DNA, "Lins" isolate. Clearly it will be possible to develop a rapid and accurate measurement of parasite numbers in whole blood through target DNA quantification.

The short pre-patent period verified in the present study, in which molecular methods were used, may have occurred by the highly inoculum concentration employed. This highly concentration was similar to those ones employed in the studies performed by Igbokwe et al. (1996) and Schenk et al. (2001). It was also observed that the PCR assay possessed an identical capacity of detection for *T. vivax* DNA as the qPCR assay (61.1%). This similarity in detection capacity could be explained by the fact that the qPCR assay described by Silbermayr et al. (2013) was directed to ITS1 region that has a high CG content, which can interfere with the diagnostic accuracy of qPCR (FIKRU et al., 2016). Fluctuations in parasitaemia and aparasitemic intervals make the diagnosis of animal trypanosomiasis challenging since it is difficult to directly detect the parasite, especially in the subpatent phase of infection (CADIOLI et al., 2015); thus, the use of more sensitive diagnostic tools such as PCR and qPCR is necessary. The detection rate of the molecular methods was higher than the parasitological methods. Both molecular techniques were able to identify 61.1% of the known positive samples, whereas

the hematocrit centrifugation technique, the best parasitological method, detected 44.4%. The molecular detection rates are similar to that described by Cadioli et al. (2015), who also used the *T. vivax CatL* target region and worked with experimentally infected cattle. Previously Tran et al. (2014) using the ITS1 as target region when working with experimentally infected cattle, obtained a detection rate of 84.9%.

Through qPCR analysis it was possible to verify that the patent phase of the illness, where parasites can be detected without interruption, may extend up to 42 DAI. This is well beyond the 14 DAI and 30 DAI previously reported by Rodrigues et al. (2013) and Fidelis et al. (2016), respectively, which used direct parasitological techniques to quantify the parasitaemia. Thus, the use of more sensitive molecular tool may help in a better understanding of the parasite dynamics during the infection. The two molecular techniques presented similar detection rates, obtaining a Kappa index of 0.87, which presented a strong agreement (LANDIS & KOCH, 1977). Although molecular techniques are effective in detecting infected animals in the patent phase of the disease, they have failed, on some occasions, to detect known positive samples during the subpatent phase. Serological tests can be useful tools for this phase of the illness, especially for cases that occurred in areas considered disease free.

High antibody levels have been described as an efficient mechanism to control parasitaemia (MATTIOLI & WILSON, 1996), which may explain the findings of the present study where all animals were seropositive from 14 DAI, for both serological techniques. The higher sensitivity of serological tests when compared to parasitological (MATTIOLI et al., 2001) and molecular tests (CADIOLI et al., 2015) suggests their use in epidemiological studies, in which the overall exposure to trypanosome infection is being investigated, and/or in cases in which no treatment was performed or non-specific anti-*Trypanosome* drugs were used. Care should be taken when using a single ELISA for *T. vivax* diagnosis, as antibodies are likely to cross-react with other parasites of the same genus (DESQUESNES, 2004). Another complicating factor is that serological tests do not indicate if the infection is active or if the animal has responded to treatment (CADIOLI et al., 2012, 2015; BOULANGÉ et al. 2017). According to Batista et al. (2007), anti-*T. vivax* antibodies in cattle that did not present clinical signs suggest the occurrence of subclinical disease, a form of the disease in which the detection of circulating parasites is difficult.

Although *T. vivax* visualization in blood smears is still the main method for the diagnosis of the disease and direct parasitological techniques are widely practiced (DESQUESNES, 2004), these methods presented the lowest sensitivity among the tests evaluated. Serological techniques are efficient in detecting seropositive animals, especially in the subpatent phase of infection, but in spite of being highly sensitive, they only demonstrate exposure to the infectious agent (DESQUESNES, 2004; OSÓRIO et al., 2008). The molecular techniques are highly sensitive, especially in the patent phase of the disease, but also demonstrated good sensitivity during the subpatent phase. They could be used in conjunction with serological techniques in the search of animals that remain infected after treatment (unpublished data). With the emergence of more sensitive and accurate molecular tools we anticipate that in the future the detection of positive animals will be improved, even in cases of low parasitaemias. However, for the present time, the combination of several diagnostic techniques can avoid the generation of false negative results, enable a better disease control and, consequently, the reduction of economic damage generated by *T. vivax* infection of productive cattle.

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