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Original Article

Follow-up of dairy cattle naturally infected by *Trypanosoma vivax* after treatment with isometamidium chloride

Acompanhamento de bovinos leiteiros naturalmente infectados com *Trypanosoma vivax* após tratamento com cloreto de isometamidium

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Abstract

Trypanosoma vivax infections cause nonspecific clinical signs in cattle associated with aparasitemic intervals, making disease diagnosis a challenge. In Brazil, diminazene aceturate and isometamidium chloride (ISM) are available to treat bovine trypanosomosis. The objective of this study was to follow-up, by molecular and serological techniques, dairy cattle naturally infected by *T. vivax* after ISM treatment. Thirty cattle naturally infected with *T. vivax* received two applications of ISM, at a dosage of 1.0 mg/kg intramuscularly, on days 0 and 150. For *T. vivax* diagnosis, EDTA-blood and serum samples were evaluated on 0, 7, 15, 30, 60, 90, 120, 150, 180, 210, and 240 days after treatment PCR, Loop-mediated isothermal amplification (LAMP) and ELISA. Animals with persistent detection of *T. vivax* DNA by both PCR and LAMP were found and continuous detection of anti-*T. vivax* IgG antibodies by ELISA, suggesting the presence of *T. vivax* resistance to ISM. The combination of LAMP and ELISA tests can prevent misdiagnosis of the parasite clearance in treated cattle, contributing to better disease control. This is the first experiment that demonstrates the persistence infection of *T. vivax* under ISM treatment in a natural infected herd and evidence of ISM chemotherapy-resistant *T. vivax* in Brazil.

Keywords: Trypanosomosis, diagnosis, serology, LAMP, clearance.

Resumo

Em bovinos, infecções por *Trypanosoma vivax* geram sinais clínicos inespecíficos que, associados a intervalos aparasitêmicos, faz com que o diagnóstico da enfermidade seja desafiador. No Brasil, somente aceturato de diaminazeno e cloridrato de isometamidum (ISM) estão disponíveis para o tratamento da tripanossomose bovina. Este trabalho teve como objetivo acompanhar bovinos leiteiros naturalmente infectados por *T. vivax*, após o tratamento com ISM por meio de técnicas moleculares e sorológica. Foram utilizados 30 bovinos naturalmente infectados com *T. vivax*, sendo estes tratados com duas aplicações de ISM, na dosagem de 1,0 mg/kg por via intramuscular profunda, nos dias 0 e 150. Foram avaliadas, para diagnóstico de *T. vivax*, amostras de sangue acrescido de EDTA e soro, colhidas nos 0, 7, 15, 30, 60, 90, 120, 150, 180, 210 e 240 dias após os tratamentos pela reação em cadeia da polimerase (PCR), amplificação circular isotérmica do DNA (LAMP) e ensaio de imunoabsorção enzimático (ELISA). Verificou-se a presença de animais com persistência na detecção de DNA de *T. vivax* pela PCR e LAMP, bem como detecção contínua de anticorpos IgG anti-*T. vivax* pelo método de ELISA, sugerindo a presença de resistência de *T. vivax* ao ISM. A combinação dos testes LAMP e ELISA pode evitar falsos diagnósticos da

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eliminação do parasita nos bovinos tratados, contribuindo para um melhor controle da doença. Este é o primeiro experimento que demonstra infecção persistente do *T. vivax* em rebanho naturalmente infectado, tratado com ISM, e evidencia possível resistência ao quimioterápico no Brasil.

Palavras-chave: Tripanossomíase, diagnóstico, sorologia, LAMP, eliminação.

Introduction

Trypanosomosis is caused by protozoa belonging to the *Trypanosoma* genus that infect domestic and wild animals. *Trypanosoma vivax, T. evansi, T. theileri* and *T. cruzi* are responsible for animal trypanosomosis in Central and South America (Jones & Dávila, 2001; Dávila et al., 2003). *Trypanosoma vivax* infects many domestic and wild ungulates; indeed, it is the principal etiological agent of trypanosomosis in cattle, being responsible for substantial economic losses in cattle rearing in South America (Seidl et al., 1999; Jones & Dávila, 2001).

The diagnosis of bovine trypanosomosis caused by *T. vivax* can be performed by clinical examination associated with parasitological, serological, and molecular methods. However, the diagnosis becomes challenging due to the lack of nonspecific clinical signs (Cadioli et al., 2012; Fidelis et al., 2016) and the presence of low parasitemia or aparasitemic periods (Fidelis et al., 2016). Serological methods, such as ELISA, represent an essential tool for screening of *T. vivax* exposure at the herds level (Cuglovici et al., 2010), but do not allow neither to discriminate animals with active infection from those treated and cured (Nantulya, 1990) nor to identify infected animals during the first days of infection (Fidelis et al., 2019). On the other hand, molecular methods, such as PCR (Cortez et al., 2009; Pimentel et al., 2012) and LAMP (Njiru et al., 2011), show a high capability for *T. vivax* DNA detection, even in periods supposedly considered as aparasitemic (Cadioli et al., 2015).

Diminazene aceturate (DA) and isometamidium chloride (ISM) are the two trypanocidal drugs licensed by the Ministry of Agriculture, Livestock, and Supply (MAPA) for *T. vivax* treatment in Brazil. However, there are reports of South American *T. vivax* strains refractory to DA in French Guiana (Desquesnes et al., 1995) and evidence of resistance in Brazil (Cadioli et al., 2012; Bastos et al., 2017). Despite the existence of reports on *T. vivax* resistance to ISM in African isolates (Giordani et al., 2016), in Brazil, until now, there is only one paper on the susceptibility of *T. vivax* in farm animals, which reports that ISM was effective in the clearance of parasites in experimentally infected cattle (Bastos et al., 2020). On the other hand, there are no studies on the susceptibility of *T. vivax* to ISM in naturally infected cattle in Brazil, as well as the use of LAMP to monitoring the parasitological cure after treatment. Thus, the present study aimed to follow-up dairy cattle naturally infected by *T. vivax* after ISM treatment, by both molecular and serological methods, to verify parasitological cure.

Material and Methods

The present study was approved by the Animal Use Ethics Committee (CEUA) of the School of Agricultural and Veterinary Studies (FCAV), São Paulo State University (Unesp), Jaboticabal, under the process number 9.277/16.

Farm characterization, clinical signs, animal selection and treatment

The Dairy farm used in this study was located in Mococa, São Paulo (21º 28' 04" S and 47º 00' 17" W), with a total area of 100 hectares. The farm herd consisted of six horses and 608 Girolando and Gir dairy cattle, vaccinated against mastitis, leptospirosis and bovine respiratory disease, clostridiosis, brucellosis, rabies and foot-and-mouth disease. For the control of worms, in non-lactating animals, there is the frequent use of endectocides doramectin, ivermectin and levamisole cloridrate. Diptera and biting flies (*Tabanus* spp., *Stomoxys calcitrans,* and *Haematobia irritans*) control were frequently performed with fipronil and cypermethrin pour on. During the experimental period, animals presented low *Stomoxys calcitrans* infestation and the absence of *Haematobia irritans* and *Tabanus* spp. Oxytocin was administrated to all animals before milking, and sharing of syringes and needles was usual before *T. vivax* was diagnosed. After trypanosomosis diagnosis, in order to reduce *T. vivax* transmission, syringes and needles sharing was abolished, and one needle and syringe per animal was used. Every equipment contaminated with bovine blood was washed with running water and detergent, then stored in a solution with alkyl dimethyl benzyl ammonium chloride and polyoxyethylene nonylphenyl ether for two hours. After this, blood-contaminated equipment was immersed in hot water (85ºC) for 20 minutes and dried, reused in the next milking.

T. vivax could have been introduced into the farm through two cows borrowed from another dairy farm located in Rifaina, São Paulo (20º 04' 50" S and 47º 25' 17" W), in early 2012, remaining there a year. Upon return, these

cows presented low body score, hyporexia, cough, and nasal discharge. One of these cows died within the first days after return. Four months after returning these animals, other animals presented progressive weight loss, a sudden drop in milk production, abortion, cough, and death. No response to the antibiotic, anti-inflammatory, and supportive therapy was observed. Over time, the occurrence of sick animals decreased, with sporadic cases leading to deaths. However, surviving cattle did not recover their previous body score and did not return to their original milk production, always being below the herd average.

Given the above, the occurrence of bovine trypanosomosis was suspected. For the initial *T. vivax* diagnosis, whole blood samples were collected in tubes containing EDTA from animals with a history of clinical signs resembling trypanosomosis. A total of 13.6% (83/608) cattle were sampled and submitted to the thick-drop test (Brener, 1961) and LAMP. Although all samples were negative for the parasitological test, 41.0% (34/83) were positive in the LAMP test. Among these, 30 samples (Bovine 1 to 30) were randomly selected to design the present study.

After diagnosis, all animals, including newborns and horses, were treated with 1.0 mg/Kg of isometamidium chloride (ISM; Vivedium®, Sumaré, CEVA Saúde Animal S.A.) intramuscularly deep in the neck, on day 0 (D0). After 150 days from the first treatment (D150), all animals received another dosage. The 30 selected animals were followed by 240 days after treatment, and blood samples were collected from the external jugular vein on days 0, 7, 15, 30, 60, 90, 120, 150, 180, 210, and 240, using evacuated collection tubes with and without EDTA. Samples from D0 and D150 were collected immediately before ISM application. Blood and serum samples were aliquoted in triplicate and stored at -80ºC until required. During the experimental period, there was no outgoing or purchase of animals. Bovine number 10 died on day 136 due to acute bleeding from udder injury, and it was not possible to collect material from D150 onwards.

Molecular assay

EDTA-whole blood DNA extraction was performed based on the genomic DNA isolation protocol described by Kuramae-Izioka (1997). In order to avoid sample contamination, DNAse and RNAse free ultra-pure autoclaved water (Invitrogen®, Carlsbad, USA), called extraction controls, was added in each extraction batch. Each DNA was subject to spectrophotometric analysis (Nanodrop 2000®, Thermo Scientific, USA), aiming at obtaining the measurement of concentrations and 260/280 and 260/230 ratios. All samples and extraction controls were subjected to PCR for the endogenous gene GAPDH, as described by Birkenheuer et al. (2003), using GAPDH F (5´CCTTCATTGACCTCAACTACAT3´) and GAPDH R (5´CCAAAGTTGTCATGGATGACC3´) primers.

PCR for *T. vivax* was performed using a set of primers based on the DNA sequence of the *T. vivax* CatL gene, as previously described by Cortez et al. (2009), using the primers (TviCatL [5´ GCCATCGCCAAGTACCTCGCCGA3´] and DT0155 [5´ TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA3´], IDT®, USA), that flank a fragment of 177 base pairs of the CatL-Like catalytic domain region gene, which encodes the *T. vivax* Cathepsin L-like (CatL) enzyme. *T. vivax* strain Lins (Cadioli et al., 2012) DNA was used as a positive control.

All animals on D0 and samples that presented Absorbance (*A*) above 0.400 in ELISA were submitted to an additional PCR assay for *T. theileri*, according to the methodology described by Rodrigues et al. (2010), using TthCatL1 (5`CGTCTCTGGCTCCGGTCAAAC3`) and DTO155 (5´TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA3´) primers. *In vitro* cultivated *T. theileri* sample was used as the positive control, and sterilized ultra-pure water (Invitrogen®, Carlsbad, USA) was used as a negative control in all PCR assays.

All PCR assays were conducted in T100™ Thermal Cycler (Bio-Rad, USA), and products were analyzed on 2% agarose gel containing ethidium bromide. The gel image was obtained using the ChemiDoc™ MP imaging system (Bio-Rad) with 100 bp size DNA standards (GeneRuler 50 bp DNA Ladder; Thermo Fisher Scientific; Waltham, MA, USA).

For the LAMP technique, a set of primers flanking a 125 base pairs fragment of the *T. vivax* satellite DNA sequence (GenBank: J03989), as described by Njiru et al. (2011), was used. Primers used were outer primers F3 (5´TGTTCTGGTGGCCTGTTGC3´) and B3 (5´GGCCGGAGCGAGAGGTGC3´), internal primers FIP (5´GTGGAGCGTGCCAACGTGGACCCGCTCCCAGACCATA3´) and BIP (5´TGTCTAGCGTGACGCGATGGAAGAGGGAGTGGGGAAGG3´), and loop primers LF (5´CACATGGAGCATCAGGAC3´) and LB (5´CCGTGCACTGTCCCGCAC3´). All reactions were performed in triplicate, accompanied by sterilized ultra-pure water (Invitrogen®, Carlsbad, USA) as negative control and *T. vivax* Lins strain DNA (Cadioli et al., 2012; Garcia et al., 2014) as a positive control. Reactions were conducted in CFX96 thermocycler (BioRad, USA) at 63°C for 60 mins, resulting in 60 cycles. After raising the temperature to 80°C/5 min., the melting curves were acquired using 0.5ºC/5 s increases for each step from 63 to 96ºC, to confirm the specificity of the amplicons for

T. vivax. Results were read through observation of amplification curves using the Bio-Rad CFX Manager software, in which the Cq (cycle threshold) of each sample was annotated.

Serological assay

The ELISA was carried out as previously described by Machado et al. (1997) and Aquino et al. (1999), with minor modifications as described below. Each microplate well (Nunc MaxiSorp®, Thermo Fischer Scientific, Massachusetts, USA) was coated with 100 µL of *T. vivax* soluble antigen at a concentration 0.1 µg/mL. All tested samples and positive and negative 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). All sera were tested in duplicate. The reaction was read by a microplate reader (MRX TC Plus, Dynex Technology, USA) at 405 nm. The blank well did not contain serum.

To define the cutoff between plates, two negative controls and one positive control were used in all test plates. Negative controls sera were obtained from two cows from a herd located in a *T. vivax* non-endemic region and previously tested using molecular (PCR and LAMP) and serological (ELISA) tests. The positive control was a bovine experimentally infected with *T. vivax* "Lins" isolate (Fidelis et al., 2016). The mean and standard deviation (SD) of the *A* from negative controls of all plates were then obtained, and the cutoff was calculated according to the following equation, described by Madruga et al. (2006):

$$\text{Cutoff} = \text{negative controls mean} + \left(3 \times \text{Negative Controls SD}\right) \quad (1)$$

Statistical analysis

The Kappa concordance test was performed between PCR, LAMP and ELISA. The test was interpreted according to Landis & Koch (1977), in which Kappa <0.00 = poor agreement; 0.00 to 0.20 = slight agreement; 0.21 to 0.40 = reasonable agreement; 0.41 to 0.60 = moderate agreement; 0.61 to 0.80 = substantial agreement; 0.81 to 1.00 = almost perfect agreement. BioEstat software version 5.0 was used for this purpose.

Results and Discussion

This study is the first to use LAMP as a diagnostic tool for the follow-up of *T. vivax* in naturally infected cattle after trypanocidal drug treatment. Also, it is the first experiment in Brazil to follow-up *T. vivax* naturally infected cattle after treatment with ISM and is a pioneer in investigating cross-reactivity by *T. vivax* and *T. theileri* in a naturally infected herd.

Molecular methods

During the experimental period, 326 EDTA-blood samples were collected. The mean and standard error (SE) of the DNA concentration, 260/230, and 260/280 ratios were 97.4 ± 4.83 ng/µL, 0.72 ± 0.02, and 1.57 ± 0.03, respectively. All samples were positive for the GAPDH gene, except for the extraction controls.

Trypanosoma vivax PCR presented five-time points of positivity, four after the first (D60, D90, D120, and D150) and one after the second (D210) ISM administration (Figure 1). All extraction controls were negative in the PCR for *T. vivax,* excluding cross-contamination during the DNA extraction procedure. Overall, PCR detected *T. vivax* DNA in only 1.8% (6/326) of samples between D0 to D240; however, in the posttreatment period (D7 to D240) PCR detected 2.0% (6/296) of positivity samples which were bovines 1 (D90), 8 (D210), 11 (D210), 20 (D120) and 5 (D60 and D150).

On the other hand, LAMP detected *T. vivax* DNA at all times, with global detection of 25.8% (84/326). Extraction controls were negative in all batches. In the posttreatment period (D7 to D240), LAMP detected the presence of *T. vivax* DNA in 18.2% (54/296) of the samples. Higher positivity was observed on D7 (9/30) and D120 (9/30). A lower positivity was observed on D60 (2/30) and D210 (2/29), 60 days after the first and second ISM applications, respectively (Table 1). The individual analysis showed fluctuation in the positivity for all animals, except bovines 7, 14, 15, 18, 23, 28, and 30, which remain negative after the first ISM treatment.

The mean and SE of the Cq of positive samples in LAMP were 39.8 ± 1.12 Cq, which characterizes samples with a low amount of *T. vivax* DNA. The DNA positive control presented an average and SE Cq of 15.4 ± 0.32. Considering

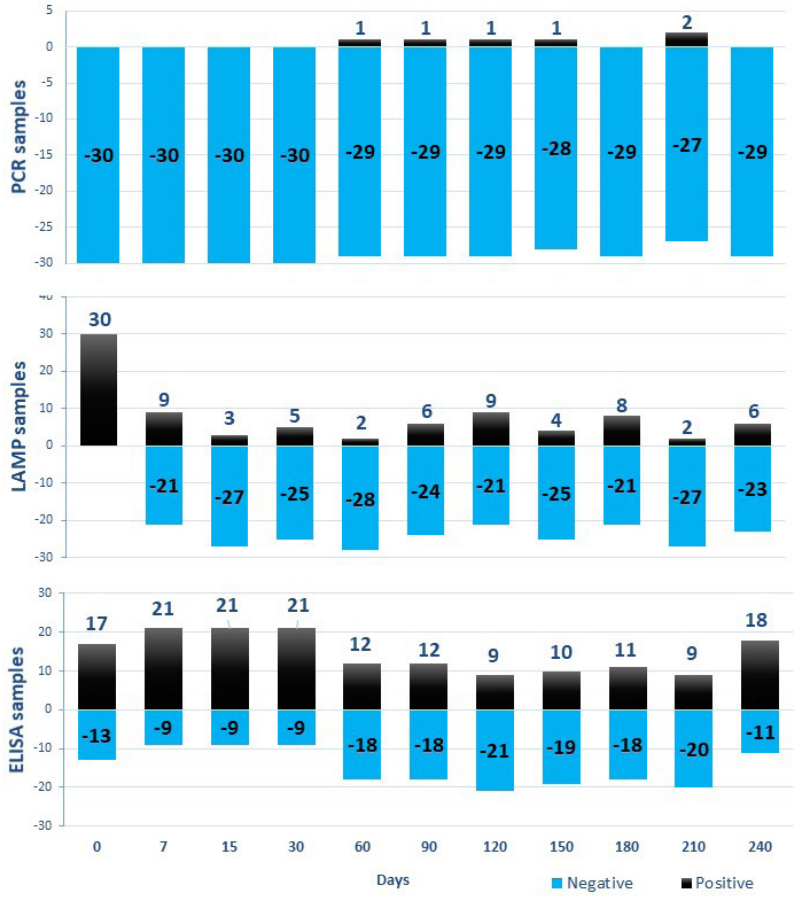

Figure 1. *Trypanosoma vivax* detection by PCR, LAMP, and ELISA techniques in blood or serum samples from naturally infected cattle before and after isometamidium chloride hydrochloride treatment (1 mg/Kg) at D0 e D150.

that the melting temperature specific for *T. vivax* DNA was 87.5ºC, samples presenting melting temperatures between 87.0 and 88.0ºC were considered positive.

LAMP is an available tool for diagnosing several pathogens, and it is increasingly used for humans (Grab et al., 2011) and animal trypanosomosis diagnosis (Haji et al., 2015). LAMP results revealed that all selected time points presented animals keeping *T. vivax* DNA after treatment. No difference between the number of positivity and days after the first (D7 to D150) or second ISM application (D180 to D240) was found.

Throughout the experimental period, except for D210, LAMP presented higher detection capacity when compared to PCR. Increased diagnostic capacity for *Trypanosoma* DNA detection was also observed by other authors when LAMP was employed (Cadioli et al., 2015; Laohasinnarong et al., 2015). The higher diagnostic capability of LAMP is because this technique has a detection threshold of 1 pg of DNA, which corresponds to 1 trypanosome/mL of blood (Njiru et al., 2011).

The use of PCR resulted in misdiagnosis in every analyzed time point, except for D210, in which the two animals detected by PCR were also positive in LAMP. Heparin, free hemoglobin, myoglobin, antibodies, and fecal contamination may inhibit PCR by inactivating *Taq* DNA polymerase (Kuboki et al., 2003; Grab et al., 2005; Schrader et al., 2012), which is less likely to occur in LAMP (Kaneko et al., 2007; Nimitphak et al., 2008), as the later uses *Bst* DNA polymerase. Herein, the presence of PCR inhibitors was discarded once all samples tested were positive for the GAPDH gene, thus ensuring the samples' quality and the integrity of the extracted DNA. Fidelis et al. (2019) showed that PCR and real-time PCR had the same detection rates for *T. vivax*; instead, qPCR is useful to determine parasitic load but much more expensive than conventional PCR.

Low concentrations of *T. vivax* DNA related to low parasitemia may also affect the PCR outcomes (Morlais et al., 2001; Cadioli et al., 2015, Fidelis et al., 2019). In the present study, LAMP positive samples presented high mean

Table 1. DNA detection dynamics of *Trypanosoma vivax* by LAMP in animals that presented detectable DNA fluctuations after treatment with isometamidium hydrochloride at a dose of 1 mg/kg in D0 and D150.

Animal	D0	D7	D15	D30	D60	D90	D120	D150	D180	D210	D240
Bovine 1	+	-	-	+	-	+	+	-	-	-	+
Bovine 2	+	+	-	-	-	-	-	-	+	-	+
Bovine 3	+	+	-	+	-	-	-	-	-	-	-
Bovine 4	+	-	-	-	-	-	-	-	-	-	+
Bovine 5	+	+	-	-	+	+	-	+	+	-	-
Bovine 6	+	-	-	-	-	-	+	-	-	-	-
Bovine 8	+	+	-	-	-	-	-	+	-	+	-
Bovine 9	+	-	-	-	-	-	-	-	+	-	-
Bovine 10	+	+	-	-	-	+	-	x	x	x	x
Bovine 11	+	-	-	-	-	-	-	-	+	+	-
Bovine 12	+	-	-	-	-	-	+	-	-	-	+
Bovine 13	+	-	-	-	-	-	-	+	-	-	-
Bovine 16	+	+	-	-	+	-	-	-	-	-	-
Bovine 17	+	-	-	-	-	+	+	-	+	-	-
Bovine 19	+	-	-	+	-	-	-	-	+	-	-
Bovine 20	+	-	-	+	-	+	+	-	+	-	-
Bovine 21	+	-	-	+	-	-	+	-	-	-	-
Bovine 22	+	-	-	-	-	-	+	-	-	-	-
Bovine 24	+	+	-	-	-	-	-	-	+	-	-
Bovine 25	+	-	+	-	-	-	+	-	-	-	-
Bovine 26	+	-	-	-	-	+	-	+	-	-	+
Bovine 27	+	+	+	-	-	-	+	-	-	-	+
Bovine 29	+	-	+	-	-	-	-	-	-	-	-

+ (Positive); - (Negative); x (lost sample).

Cq (39.4), which indicates that positive animals experienced low *T. vivax* parasitemias after treatment, thus justifying PCR failure. Studies previously performed by Cadioli et al. (2015) and Fidelis et al. (2019) showed that in the chronic/sub-patent phase of infection, *T. vivax* tends to present very low parasitemia, which difficult disease diagnosis in herds.

Thus, LAMP has proved to be a handy tool in monitoring *T. vivax* clearance by infected animals, identifying positive animals over time, and proving a sensitive diagnostic test. The same cannot be stated for PCR, which was not able to detect a large number of positive samples, even before treatment (D0). This failure may induce errors in the herd treatment, since it is assumed that treatment was effective, when, in fact, the technique sensitivity is low. Thus, PCR contributes to the maintenance of "apparently" cured animals in the herds, which keeps the parasite on the farm and also promote its spread through commercial routes, being considered the primary way of parasite dispersal (Silva et al., 1998; Jones & Dávila, 2001).

Fluctuations in *T. vivax* DNA detection were observed during the experimental period, which might be explained by the parasite's transient elimination, which corroborates previous observations described by other authors after ISM treatment, either by using parasitological (Dagnachew et al., 2015) or molecular diagnostic techniques (Vitouley et al., 2012). Alternatively, the amount of *T. vivax* DNA might have been below the limit of detection. The occurrence of animals presenting *T. vivax* recirculation after ISM treatment is essential information for disease

control, which can be considered as a persistent infection. In fact, if new infections by another *T. vivax* strain had occurred, positive animals in serological and molecular tests will have increased, a fact not observed in this experiment. Therefore, these observations indicate that ISM is not capable of eliminating *T. vivax* in few doses and longtime treatment will be necessary, just like keep constant herd monitoring, thus avoiding reinfections that could generate substantial impacts by treatment failure.

Serological assay

The mean and SD (SE) of *A* from the negative controls were 0.117 ± 0.031 (0.009), and the cutoff value was 0.209. Positive control presented mean and SD (SE) of 1.079 *A* ± 0.148 (0.060), respectively. Overall, ELISA detected 49.4% (161/326) of seropositive samples for *T. vivax*. It was also observed that 48.7% (144/296) of the samples were seropositive for *T. vivax* after treatment (D7 to D240).

Although the presence of seropositive animals was detected in all moments after treatment, it is possible to observe that after the first ISM application, there was a decrease in the number of seropositive animals, with the smaller number on D120 (Figure 1). It is also noted a reduction in the average values of *A*, being close to the cutoff, and reaching lower values on D60 and D150 (Figure 2). However, the second application ISM (D150), did not affect the number of seropositive animals. Indeed, an increase in *A* values was observed on the last day (D240) (Figures 1 and 2).

The lack of detectable IgG antibodies in positive animals (D0) occurred in five moments, namely D7, D15, D60, D120, and D180. Bovine one became seronegative on D7; bovine three on D15; bovines 7, 11, 17, 22, and 27 on D60; bovines 5, 20 and 21 on D120 and finally bovine 18 on D180. Diagnostic fluctuations during the experimental period was observed in bovines 1, 2, 3, 4, 5, 6, 7, 8, 11, 14, 15, 18, 19, 20, 21, 24 and 26 (Table 2). After becoming seronegative on D60, bovines 17, 22 and 27, remained so until the last evaluation. Bovines 10, 12, 16, 25, 29, and 30 remained seropositive in the entire period, and bovines 9, 13, 23, and 28 remained seronegative throughout the experimental period.

ELISA is a widely used technique for seroepidemiological studies for *T. vivax* in Africa (Dagnachew & Bezie, 2015) and South America (Osório et al., 2008; Sampaio et al., 2015), providing valuable information about herd status. In the present study, although this method diagnosed only 17 out of 30 animals considered positive by LAMP on DO (Figure 1), this serological assay showed a high capacity to detect positive animals in the posttreatment period.

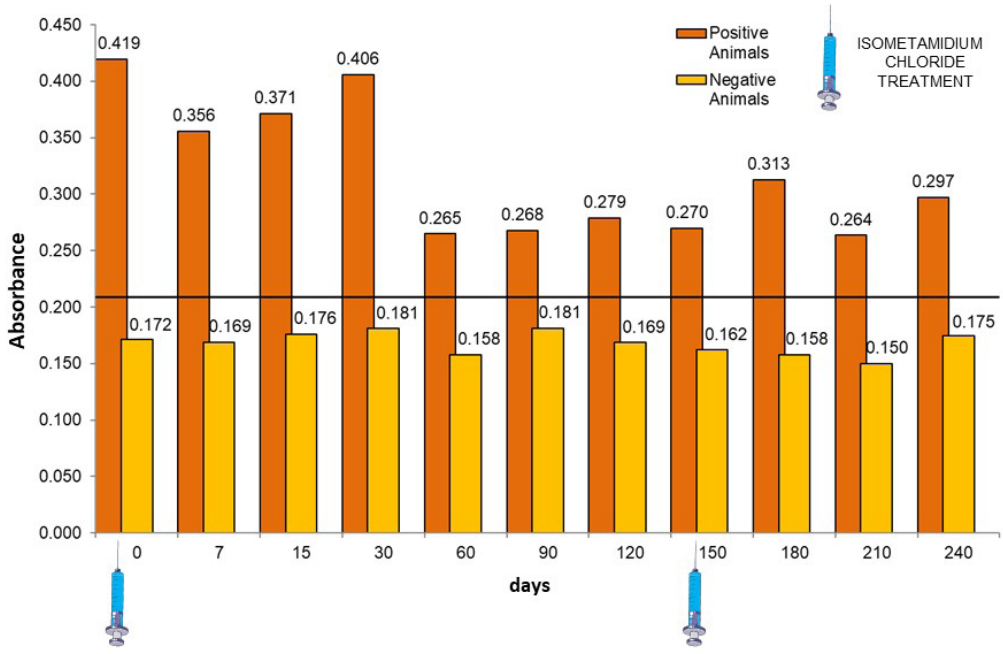

Figure 2. Anti-*Trypanosoma vivax* IgG antibodies Absorbance (*A*) in each selected time point of *T.vivax*-naturally infected cattle serum samples before and after isometamidium chloride hydrochloride treatment (1 mg/Kg) at D0 e D150.

Table 2. Dynamics of detection of anti-*Trypanosoma vivax* antibodies by ELISA in animals that presented diagnostic fluctuations after treatment with isometamidium hydrochloride at a dose of 1 mg/kg in D0 and D150.

Animal	D0	D7	D15	D30	D60	D90	D120	D150	D180	D210	D240
Bovine 1	+	-	-	-	-	-	-	-	-	-	+
Bovine 2	-	+	+	+	+	-	-	-	-	-	-
Bovine 3	+	+	-	-	-	-	-	+	+	+	+
Bovine 4	-	+	+	+	-	-	-	-	-	-	-
Bovine 5	+	+	+	+	+	+	-	-	-	-	+
Bovine 6	-	-	-	-	-	+	+	+	+	+	+
Bovine 7	+	+	+	+	-	-	+	-	+	-	+
Bovine 8	-	-	-	-	-	-	-	-	+	-	+
Bovine 11	+	+	+	+	-	-	-	+	+	-	+
Bovine 14	-	+	+	+	-	-	-	-	-	-	+
Bovine 15	-	+	+	+	-	-	-	-	-	-	-
Bovine 18	+	+	+	+	+	+	+	+	-	-	+
Bovine 19	-	-	+	+	-	-	-	-	-	-	+
Bovine 20	+	+	+	+	+	+	-	-	-	-	+
Bovine 21	+	+	+	+	+	+	-	-	-	+	+
Bovine 24	-	-	-	-	+	+	-	-	-	-	-
Bovine 26	-	+	+	+	-	-	-	+	+	+	+

+ (Positive); - (Negative).

According to Nantulya (1990), ELISA is highly sensitive for *T. vivax* diagnosis, but there are reports of unsatisfactory results, showing lower sensitivity when compared to PCR (Desquesnes, 1997) and LAMP (Cadioli et al., 2015).

Over time, antibody levels tend to decrease progressively after treatment. Desquesnes et al. (2003) and Monzon et al. (2003), using *T. vivax* and *T. evansi* total soluble crude antigens, observed that decrease occurs from 2 and 2.3 months in cattle and horses naturally infected by *T. vivax* and *T. evansi* after treatment with DA and quinapyramine sulfate, respectively. In the present study, six animals remained seropositive until the end of the experimental period, and for those that became seronegative, this occurred 60 days after the first application of ISM. This antibody behavior was also observed in cattle experimentally infected by *T. vivax* (Desquesnes et al., 2003; Pillay et al., 2013). Pillay et al. (2013), using *T. vivax* recombinant GM6 antigen, noted that most animals became seronegative 30 days after DA treatment. On the other hand, Desquesnes et al. (2003), using *T. vivax* total soluble crude antigen, found a higher number of seronegative animals 90 days after DA treatment.

On D7 was a striking reduction in LAMP detection, but by ELISA, the seropositivity and the average *A* decay occurred from D60 (Figures 1 and 2). A similar trend was observed by Bengaly et al. (2001) using molecular (PCR) and serological (ELISA) diagnostic methods to evaluate the therapeutic efficacy of DA in sheep experimentally infected by *T. vivax* and *T. congolense*. In that experiment, negativity by PCR was observed one to two days after treatment, much earlier than 60 to 100 days observed by ELISA. This late trend of antibody reduction after treatment showed by ELISA indicates that this method should be evaluated for a long time or interpreted in conjunction with molecular techniques, thus avoiding errors in assessing the actual herd infection situation.

ELISA techniques are designed to detect antibodies against *Trypanosoma* spp. These techniques are often performed using a soluble crude lysate of trypanosome antigens, which makes ELISA a problematic test to standardize and with the possibility of presenting cross-reactions with other trypanosome species, including *T. theileri* (Luckins, 1977; Desquesnes et al., 2001; Jones & Dávila, 2001). *Trypanosoma theileri* is a cosmopolitan parasite that, if not associated with other concomitant infections or stressful conditions, is not pathogenic to cattle and buffaloes. This species of *Trypanosoma* has already been detected in healthy cattle in São Paulo, Brazil (Rodrigues et al., 2003).

In order to rule out the occurrence of *T. theileri* among animals from the present study and the possibility of serological cross-reaction in the used ELISA, a PCR specific for this parasite was performed in 56 selected samples from all animals on D0 (30 samples) and 26 samples that presented ELISA *A* above 0.400. All tested samples were negative, thus ruling out the occurrence of serological cross-reaction in the ELISA.

While Kappa results between LAMP and PCR (0.10) and LAMP and ELISA (0.02) indicate slight agreement, it showed a poor agreement (-0.02) between PCR and ELISA. These results agree with those obtained by Fidelis et al. (2019), which evaluated different techniques for disease diagnoses, obtaining a Kappa index of 0.07 between PCR and ELISA. The low Kappa values observed in the present study represent a reflection of the different detection rates obtained, which are inherent to different detection targets of each technique. This finding reinforces the need for a combination of serological and molecular assays to diagnose naturally infected *T. vivax* herds, an observation already pointed out by Fidelis et al. (2019).

In Africa, *T. vivax* recirculation in cattle treated with ISM has been reported with increasing frequency (Dagnachew & Bezie, 2015) and have been associated with the long-term use of the drugs, underdosing, and formulations with inadequate chemotherapy concentrations (Dagnachew et al., 2015; Tekle et al., 2018). Moreover, parasite recirculation after treatment was correlated with the presence of *T. vivax* in refuge sites, such as cerebrospinal fluid, choroid plexus, and aqueous humor (Whitelaw et al., 1988; Batista et al., 2011), in which the drug does not reach adequate concentrations even during the prophylaxis period (Giordani et al., 2016), which could be up to 160 days for 1 mg/kg of ISM (Toro et al., 1983). Therefore, it can be hypothesized that the persistence of *T. vivax* DNA showed by the used molecular methods could be due to parasites leaving their refuge sites and returning to the bloodstream.

On the other hand, ELISA results showed seropositivity at all selected time points, with the lowest percentage of detection represented by 30% (9/30) of sampling (Figure 1) and the presence of 6 animals seropositive at all times, even after the second ISM dose. These findings indicate the occurrence of *T. vivax* antigens stimuli, possibly coming from the selection of parasites by the treatment, which may have culminated with the maintenance of animal infection.

Trypanocidal drug resistance testing can be performed by *in vivo*, *in vitro*, or by molecular methods. *In vivo* tests, such as the use of cattle in herds, is a method that does not require parasite isolation and is proposed to evaluate probable resistance in the field directly, and results are only indicative of resistance (Giordani et al., 2016). However, in the field, reinfection of animals after treatment, especially in areas with high exposure to mechanical or biological vectors, may interfere with the obtained results, mainly when drugs without prophylactic effect are used (Moti et al., 2015).

After *T. vivax* diagnostic, preventive measures were implemented in the farm to avoid the introduction of new animals in the herd, control of hematophagous flies and, treatment of all herd with ISM, including horses. Fomites as blood-contaminated needles, syringes, palpation gloves and, identification equipment were disinfected as previously commented.

Fomites control, a low infestation of blood-sucking flies, herd isolation in association with very low or apasitemic positive bovines reduces the probability of transmission between animals in the herd, so *T. vivax* detection during the experimental period allows us to state that there was no reinfection; however, the animals remain persistently infected by *T. vivax*. Considering persistent infection status as a critical problem in infected herds, treatments in intervals of 150 days may not be adequate because a raise of positive samples were detected by LAMP as observed; thus, shorten treatment intervals as 120 days must be considered.

Another relevant information related to the persistent infection observed is the possible induction of ISM resistance, which is a disturbing fact, as it may imply in treatment failure of herds when ISM is used.

Conclusions

As far we know, this is the first experiment that demonstrates the persistence infection of *T. vivax* under ISM treatment in a natural infected herd and evidence of ISM chemotherapy-resistant *T. vivax* in Brazil. The combination of both LAMP and ELISA proved to be very useful for achieving the diagnosis and monitoring of cattle naturally infected by *T. vivax* before and after treatment with ISM. On the other hand, PCR is not indicated for *T. vivax* diagnosis in cattle as a single diagnostic tool. We observed that the prophylactic dose of ISM does not eliminate *T. vivax* and treatment intervals as 120 days must be considered, an essential data of future strategies to tackle bovine trypanosomosis in Brazilian herds.

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