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Communication

[*Comunicação*]

First detection of *Trypanosoma vivax* in dairy cattle from the northwest region of Minas Gerais, Brazil

[*Primeira detecção de* Trypanosoma vivax *em bovinos leiteiros da região noroeste de Minas Gerais, Brasil*]

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Bovine trypanosomiasis is caused by the flagellated protozoan *Trypanosoma vivax*. It is biologically transmitted only in the African continent by the flies of the genus *Glossina* spp. It was brought to the American continent by European colonists in the 19th century, and although its main mechanical vector was a dipteran of the genus *Tabanus* spp. (Costa, 2018; Bastos *et al*., 2017), other dipterans such as *Stomoxys calcitrans* and *Haematobia irritans* also cause mechanical transmission (Cadioli *et al*., 2012). However, sharing of needles and syringes, especially for oxytocin administration in dairy cattle, is associated with a greater risk of spread of the protozoan in herds (Bastos *et al*., 2017).

Bovine trypanosomiasis is clinically defined as an anemia-causing disease accompanied by a decrease in body score, reduced productivity, apathy, and lethargy. The clinical signs of trypanosomiasis are similar to those of other diseases, and this hinders its conclusive diagnosis. The signs include fever, anemia, weight loss, reduced production rates, prolonged decubitus, abortion, and death (Dagnachew and Bezie, 2015). Trypanosomiasis is one the most relevant types of hemoparasitosis in tropical dairy cattle farming. It causes economic losses directly because of the fall in milk production and increased mortality, and indirectly because of the cost of diagnosis, treatment, and sanitary measures to control the disease (Abrão *et al*., 2009).

The occurrence of outbreaks in non-endemic regions is manifested in the form of more evident clinical signs and a high mortality rate (Batista *et al*., 2007; Rodrigues *et al*., 2013). This study was motivated by the lack of clinical, epidemiological, and diagnosis data of trypanosomiasis in dairy cattle in the municipality of Unaí, northwestern region of Minas Gerais, Brazil. It also aimed at clarifying the entry of the agent in herds by correlating the constant trade of animals with endemic regions and the producers' report on animals with reproductive problems.

The study was submitted to the Council for Ethics in the Use of Animals (CEUA) of the Federal University of Jequitinhonha and Mucuri Valleys (UFVJM) and is in accordance with the ethical principles and standards of the National Council for Animal Experimentation (CONSEA), being approved under protocol Nº060/2016.

Samples of whole blood and blood serum were collected from animals from five dairy farms located in Unaí, northwest mesoregion of Minas Gerais (MG), Brazil. The farms were selected for convenience based on the observations of the breed and the clinical suspicions of field

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veterinarians regarding the incidence of trypanosomiasis. Thus, Farm A (latitude 16º 29' 37.5" South, longitude 46º 52' 50.6" West, and altitude of 598m), Farm B (latitude16º 32' 14.2" South, longitude 46º 46' 58.4" West, and altitude 589m), Farm C (latitude 16º 17' 45" South, longitude 46º 58' 09" West, and altitude 577m), Farm D (latitude 16º 15' 47" South, longitude 46º 41' 31" West, and altitude 613 m), and Farm E (latitude 16 ° 23' 26" South, longitude 47º 06' 33" West, and altitude 996m), located in the city of Unaí, state of Minas Gerais, Brazil, were selected for the study.

A sectional study was conducted between 2018 and 2019. Based on convenience, 27 animals from Farm A, 29 animals from Farm B, 19 animals from Farm C, 20 animals from Farm D and 20 animals from Farm E were chosen, totaling 115 cattle. Animals that exhibited any clinical signs consistent with the disease (anemia, jaundice, progressive weight loss, drop in milk production, low fertility, and/or neurological signs) or belonged in the same batch of suspected animals, were selected to participate of the study. The selected animals were between 3 and 8 years old, and a body condition scoring of 3 to 4.5.

The selected farms had Holstein and Girolando cattle, which were kept on the pasture in the rainy season, semi-confined in the drought, or confined throughout the year (Compost Barn and Freestall). The animals received food concentrate in the trough to complement the total diet. Calf breeding took place through collective, hut, and Argentine systems. The farms had a vaccination schedule for Foot and Mouth Disease and Brucellosis, sporadically Leptospirosis, IBR (infectious bovine rhinotracheitis), and BVDV (bovine viral diarrhea virus). Furthermore, all farms used oxytocin on some or all the lactating animals daily. Oxytocin was generally used in Girolando cattle at 1 or 2 IU per animal/milking.

Blood samples were obtained aseptically by jugular venipuncture and collected into a vacutainer tube containing 10% K3-EDTA for LAMP and hematological analysis, and 10 mL was collected into an anticoagulant-free vacutainer tube for serological test. All the samples collected were immediately packaged and transported in a cool box without ice to the laboratory for processing within 3h.

Capillary tubes were filled three-quarters with blood containing 10% K3-EDTA for hematocrit analysis (Woo's technique) (Woo, 1970). Then, they were centrifuged at 10,000 rpm for 5 min, and the reading was performed using an optical microscope with a 400× magnification lens. The trypomastigote forms of *T. vivax* were observed in the plasma portion, just above the leukocyte layer.

In addition, two types of blood smears were performed to *Trypanosoma* spp. detection, thin blood smear and the concentrated thick blood smear. Both blood smears were fixed with methanol for 5 min and stained with Giemsa.

Enzyme-linked immunosorbent assay (ELISA) was performed as described by Aquino *et al*. (1999), with modifications. Each microplate well (Nunc MaxiSorp®) was coated with 100 µL of the soluble antigen at a concentration of 400 ng/mL. All samples and controls were diluted 1:50 in phosphate-buffered saline with 0,05% Tween-20, pH 7.4. The blankwell did not contain serum.

Six positive and six negative controls were used in the ELISA. Positives controls were isolated from an outbreak in Lins, SP and negatives controls from a non-endemic area in Jaboticabal, SP. Sera were tested in duplicate, and positive and negative controls were tested in quadruplicate.

The reaction was read by a microplate reader (MRX TC Plus, Dynex Technology, USA) at 405 nm. The cut-off was calculated as described by Madruga *et al*. (2006).

The samples of the animals tested positive in the serological test were submitted to a molecular test. The QIAamp DNA blood mini kit (Qiagen, Madison, United States) was used to extract the DNA from the samples, following the manufacturer's recommendations.

The Loop-mediated isothermal amplification (LAMP) assay was carried out as described elsewhere (Njiru *et al*., 2011) using a set of primers namely outer primers:
F3 (TGTTCTGGTGGCCTGTTGC) and
B3 (GGCCGGAGCGAGAGGTGC);
inner primers: FIP

(GTGGAGCGTGCCAACGTGGCACCCGCTC CCAGACCATA) and
BIP
(TGTCTAGCGTGACGCGATGGAAGAGGGA GTGGGGAAGG); and
loop primers:
LF (CACATGGAGCATCAGGAC) and
LB (CCGTGCACTGTCCCGCAC)
(IDT – Síntese Biotecnologia, Brazil).

The LAMP reactions were performed in 25µL reactions volume containing 5pmol of the outer primers, 20 pmol of loop primers, 40 pmol of the inner primers, 4 mM of extra $MgSO_4$ (New England Biolabs, MA, USA), 1 M betaine (Sigma–Aldrich, St. Louis, MO, USA) and 2.5mM deoxynucleotide triphosphates mix (dNTP – Invitrogen, USA). The 1X ThermoPol reaction buffer (New England BioLabs, MA, USA) contained 20mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$ and 0.1% Triton X-100 was used. In addition, 1µL (8 units) of Bst DNA polymerase (Large fragment; New England Biolabs) and SYTO-9 fluorescence dye at 1.5 M (Molecular Probes, OR USA) were also used. Finally, 2.5µL of each DNA sample was added as a template for each LAMP reaction.

The reactions were carried out 63ºC for 60 minutes, using a QuantioStudio 3 Thermal Cycler (AppliedBiosystems). The reaction was terminated by increasing the temperature to 80ºC for 5 min. The melting curves were acquired using 0.5ºC steps, with holds of 5 s, from 63 to 96ºC. The results were assessed through observation of amplification curves using the QuantStudio 3 software. All the Cq (quantification cycles) of each sample were annotated. Each LAMP assay was performed including triplicates of each cattle blood-DNA sample. DNA of *T. vivax* (Cadioli *et al*., 2012) and ultra-pure water were used as positive and non-template controls, respectively, in all LAMP assays.

Woo's techniques revealed the presence of protozoa in 7.4% (2/27) of the animals evaluated only in the herd on Farm A. The number of trypomastigote forms observed in the positive animals using the Woo technique ranged from 1 to 10. However, no parasite was observed by blood smears in all Farms.

Serological test revealed that 5,22% (6/115) of the animals were positive. ELISA showed positive results in three animals in Farm A (11.11%) and three in Farm B (10.34%), considering the cut-off 0.375 (Table 1). The optical density readings of the positive samples were 0.515, 0.426, 0.527, 0.448, 0.498 and 0.803.

Table 1. Frequency of animals positive for *Trypanosoma vivax*, detected by parasitological (thick drop, blood smear, Woo), serological (ELISA), and molecular (LAMP) examination in cows belonging to dairy farms in the municipality of Unaí, Minas Gerais, Brazil, 2018 - 2019

Farms	Drop Thick	Woo	Smear	ELISA	LAMP	Total Positive	Total Analyzed
Farm A	0	2	0	3	3	3	27
Farm B	0	0	0	3	2	2	29
Farm C	0	0	0	0	0	0	19
Farm D	0	0	0	0	0	0	20
Farm E	0	0	0	0	0	0	20
TOTAL	0	2	0	6	5	5	115

The LAMP molecular diagnosis identified 83.33% (5/6) positive animals among those who had already tested positive in the serological test. Of the animals tested positive in the LAMP, three belonged to Farm A (60.0%) and two to Farm B (40.0%). The Cq of the positive samples ranged from 44 to 51, while the positive control cq was 31. Additionally, the melting temperature

(TM) obtained among the positive samples varied from 88.18 °C to 88.5 °C. Finally, the positive control TM was 88.05 °C.

The present study reports the first detection of *T. vivax* in cattle from dairy farms in the Northwest Region of Minas Gerais, Brazil. The clinical fact reported by the visited owners was the low reproductive rate, observed through the repetition of heat, low conception rate, and increased interval between births of the animals.

The animals selected for this study belonged to batches of cows with a conception rate of <30% in the last year. Despite this, one of the animals that tested positive for *T. vivax* (positive in the Woo, serological, and LAMP techniques) produced highest the amount of milk in the herd during the study. The other animals that tested positive for *T. vivax* showed average milk production. The positive animals had a good body score and adequate milk production curve for the lactation period.

A similar study performed on 24 farms distributed across 14 municipalities in the state of Goiás, Brazil (Bastos *et al*., 2020), revealed an average prevalence of 8.84% and high parasitemia levels by *T. vivax.*

Farm A obtained the largest number of positive samples, 11.11% (03/27), with one animal testing positive in the parasitological (Woo), serological (ELISA), and molecular (LAMP) tests. It is possible that there is not always a correlation between the levels detected by serological assay and a molecular detection of *Trypanosoma vivax* in chronically infected animals since the chronic phase of bovine trypanosomosis may be due to the mechanisms of evasion of the host's system. Alterations in the expression of variable surface glycoproteins will lead to antigenic mutation, rendering defense antibodies previously produced by the host ineffective. Thus, this immune evasion mechanism will lead to fluctuations in parasitemia, making it difficult to eliminate the parasite, causing the infection to become chronic (Baral, 2010). Additionally, trypanosomatids can internalize antibodies that bind to their surface, contributing to an efficient evasion of the immune system (Hill *et al*., 2005).

Serological tests have high sensitivity when compared with parasitological and molecular tests (Mattioli *et al*., 2001; Cadioli *et al*. 2015). Fidelis Junior *et al*. (2019) evaluated 54 samples known to be positive for *T. vivax* and observed the following results: parasitological 44.4%, molecular 61.1% and serological ELISA 90.7% (49/54) and RIFI 94.4% (51/ 54). Thus, tests such as ELISA or RIFI are highly indicated for use in investigations that aim to identify the presence of the agent in herds. However, in treated herds, care should be taken when using serological techniques for the diagnosis of *T. vivax*, since it would not be possible to differentiate between active infection and treatment-responsive animal (Boulangé *et al*. 2017). In addition, it is necessary to consider the possibility, even if low, of cross-reactions with other *Trypanosoma* species such as *Trypanosoma evansi* and *Trypanosoma theileri* (Madruga *et al*., 2006).

Other studies have already described reproductive problems and high rates of abortions in animals infected with *T. vivax* in other Brazilian regions (Vieira *et al*., 2017; Bastos *et al*., 2017). The economic importance of the infection, even if it does not cause evident clinical signs, is demonstrated by the great losses caused to milk production because milk production depends on efficient reproduction. These reports differ from most clinical signs characteristic of the disease, such as marked weight loss, decreased production, apathy, anorexia, enlarged lymph nodes, nervous symptoms, and death (Dagnachew & Bezie, 2015). Animals with acute anemia, apathy, anorexia, weight loss, and severe reduction in milk production were commonly reported in outbreaks of trypanosomiasis in the state of Minas Gerais (Reis *et al*., 2019).

Although the present study detected the circulation of *T. vivax* in cattle from dairy farms in the municipality of Unaí, animals with classic clinical signs of trypanosomiasis were not observed. Carvalho *et al*. (2008), when evaluating the first outbreak of bovine trypanosomiasis in Minas Gerais, and Silva *et al*. (1996), when evaluating it in Mato Grosso, observed that the animals had fever, anorexia, enlarged lymph nodes, apathy, rapid weight loss, abortion, and diarrhea and died. In an outbreak that occurred in the municipality of Itambé - PE,

27.5% of the dairy cows had fever, anorexia, pale mucous membranes, diarrhea, reduced milk production, premature births, and a high number of abortions (Pimentel *et al*., 2012). In São Paulo, cattle affected by *T. vivax* had fever, jaundice, decreased milk production, weight loss, reactive lymph nodes, submandibular edema, diarrhea, and abortions and died (Cadioli *et al*., 2012). In a recent experimental study, calves inoculated with *T. vivax* had apathy, anorexia, hyporexia, diarrhea, and hyperthermia (Bastos *et al*., 2020).

Trypanosoma vivax may have been introduced into the bovine herd in the municipality of Unaí-MG through animal trade with other mesoregions of Minas Gerais where the agent circulates endemically. The entry of animals from other regions, seeking to improve the genetics of the herd, in addition to opening doors for increased production and economic capital, also allows the entry of new diseases that did not exist in the region before. In the state of Rio de Janeiro, the entry of *T. vivax* infection in cattle was probably caused by the trade of animals from endemic areas of Brazil (Costa, 2018). The risks of transporting infected animals had been previously reported in Paraíba (Batista *et al*., 2008), Minas Gerais (Carvalho *et al*., 2008; Abrão *et al*., 2009; Cuglovici *et al*., 2010), and São Paulo (Cadioli *et al*., 2012).

Factors such as the presence of blood-sucking flies and use of intravenous, intramuscular, and subcutaneous medications with shared needles and syringes are most likely causes of the spread of the agent within the herd. Hematophagous flies belonging to the genera *Stomoxys* spp. and *Tabanus* spp. are some of the probable mechanical transmission vehicles (Costa, 2018). Although blood-sucking arthropods may have an important role in the epidemiology of the disease, flies of the genera *Stomoxys* spp. and *Tabanus* spp. are rarely reported in cattle herds in the region where *Haematobia irritans* prevails. Thus, the use of oxytocin in the management of lactating cows, owing to the Girolando cattle present in the region, is most likely what causes the agent to spread within the herds. The correlation between the occurrence of an outbreak of trypanosomiasis in dairy cattle receiving oxytocin twice a day and the sharing of needles and syringes was reported in Monte Carmelo, MG (Souza *et al*., 2019) and Patos de Minas, MG (Germano *et al*., 2017).

T. vivax circulates in the dairy cattle herds in the municipality of Unaí, northwest of the state of Minas Gerais. However, the analyzed animals did not manifest the characteristic clinical signs of the disease, and the manifestation was characterized by reports of reproductive problems.

Keywords: bovine trypanosomiasis, ELISA, LAMP

RESUMO

A tripanossomíase bovina é causada pelo protozoário Trypanosoma vivax. *A transmissão biológica ocorre apenas no continente africano pela mosca Tsé-tsé, de forma mecânica por dípteros hematófagos em todos os continentes, ou pelo compartilhamento de agulhas e por práticas associadas. O estudo teve como objetivo relatar o primeiro diagnóstico parasitológico, sorológico e molecular de* T. vivax *em bovinos leiteiros provenientes de cinco propriedades do município de Unaí, Minas Gerais, Brasil. Cento e quinze animais selecionados por conveniência apresentavam sinais clínicos ou pertenciam a lotes de animais suspeitos. Foram detectados positivos pelos testes parasitológico (técnica de Woo), sorológico (ELISA) e molecular (LAMP). A maior prevalência global para* T. vivax *foi de 11,11% na propriedade A. O único sinal clínico dos animais positivos estudados foi baixa taxa de concepção. O primeiro diagnóstico de tripanossomíase no noroeste mineiro é extremamente importante, haja vista o tamanho do rebanho leiteiro da região e as possíveis perdas econômicas provocadas pela enfermidade. Ademais, faz-se necessário maior controle sanitário na região, uma vez que a transmissão no Brasil é intimamente ligada às práticas de compartilhamento de agulhas no manejo dos animais e ao parasitismo de moscas hematófagas.*

Palavras-chave: tripanossomíase bovina, teste ELISA, LAMP

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