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Detection and molecular characterization of *Trypanosoma* (*Duttonella*) *vivax* in dairy cattle in the state of Sergipe, northeastern Brazil

Detecção e caracterização molecular de *Trypanosoma (Duttonella) vivax* em gado leiteiro no estado de Sergipe, no Nordeste do Brasil

Osires Lustosa Eloi Vieira[1]; Lucia Oliveira de Macedo[1]; Marcos Antônio Bezerra Santos[1]; José Augusto Bastos Afonso Silva[2]; Carla Lopes de Mendonça[2]; Maria Aparecida da Gloria Faustino[3]; Carlos Alberto do Nascimento Ramos[4]; Leucio Câmara Alves[3]; Rafael Antonio Nascimento Ramos[1]; Gílcia Aparecida de Carvalho[1]*

[1]Unidade Acadêmica de Garanhuns – UAG, Universidade Federal Rural de Pernambuco – UFRPE, Garanhuns, PE, Brasil

[2]Clínica de Bovinos de Garanhuns, Universidade Federal Rural de Pernambuco – UFRPE, Garanhuns, PE, Brasil

[3]Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco – UFRPE, Recife, PE, Brasil

[4]Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, MS, Brasil

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Abstract

Trypanosoma (*Duttonella*) *vivax* is an important cause of economic losses among feedlot cattle. These losses are related to the morbidity, mortality, reproductive issues and decreased production. It is known that the clinical signs observed in infections by this protozoon are similar to other hemoparasitosis, which difficult the diagnosis. Therefore, the aim of this study was to detect and molecularly characterize an outbreak of trypanosomiasis caused by *T.* (*D.*) *vivax* in dairy cattle in the municipality of São Miguel Aleixo, state of Sergipe, Brazil. Blood samples from cattle (n = 15) presenting clinical signs compatible with trypanosomiasis were collected and parasitological and molecular evaluated. Among the samples analyzed, 34% (5/15) were positive from blood smears, 60% (9/15) from the buffy coat method and 80% (12/15) from the molecular method. The DNA sequence obtained (659 bp) showed 99% similarity to *T.* (*D.*) *vivax* sequences that are available in the GenBank database. The presence of this protozoon in cattle herds is a problem for producers. Diagnosing trypanosomiasis is problematic because its evolution is similar to that of other parasitic blood diseases. In addition, this is the first report of infection by *T.* (*D.*) *vivax* in cattle in the state of Sergipe, northeastern Brazil.

Keywords: Trypanosomiasis, parasitological diagnosis, molecular diagnosis.

Resumo

Trypanosoma (*Duttonella*) *vivax* é responsável por consideráveis perdas econômicas na bovinocultura. Estas perdas estão relacionados à morbidade, mortalidade, problemas reprodutivos e declínio na produção. Sabe-se que os sinais clínicos apresentados em infecções por este protozoário se assemelha a outras hemoparasitoses, dificultando muitas vezes o diagnóstico. Portanto, objetivou-se com este estudo detectar a ocorrência de *T.* (*D.*) *vivax* em bovinos leiteiros no município de São Miguel Aleixo, Estado de Sergipe, Brasil. Para tanto, amostras de sangue (n = 15) foram coletadas e avaliadas através de métodos parasitológicos e moleculares. Do total das amostras analisadas, 34% (5/15) foram positivas no esfregaço sanguíneo, 60% (9/15) pelo método do Buffy Coat, enquanto na biologia molecular 80% (12/15) amplificaram um fragmento de DNA (659 pb) compatível com *T.* (*D.*) *vivax* (GenBank). Em conclusão a presença de *T.* (*D.*) *vivax* nos rebanhos bovinos caracteriza-se como um problema para os pecuaristas, como também para o diagnóstico, uma vez que essa tripanossomíase apresenta evolução semelhante a outras hemoparasitoses. Ademais, este é o primeiro relato de infecção por *T.* (*D.*) *vivax* em bovinos do estado de Sergipe, nordeste do Brasil.

Palavras-chave: Tripanossomíase, diagnóstico parasitológico, diagnóstico molecular.

***Corresponding author:** Gílcia Aparecida de Carvalho. Unidade Acadêmica de Garanhuns – UAG, Universidade Federal Rural de Pernambuco – UFRPE, Av. Bom Pastor, s/n, Boa Vista, CEP 55292-270, Garanhuns, PE, Brasil.
e-mail: gilcia.acarvalho@yahoo.com

Introduction

Trypanosomiasis is a parasitic infection caused by protozoa belonging to the genus *Trypanosoma*, which affects domestic and wild mammals, including humans (GARDINER, 1989). Species such as *Trypanosoma brucei*, *T. congolense*, *T.* (*Duttonella*) *vivax* and *T. evansi* cause important economic losses in animal herds (HOARE, 1972), while *T. cruzi* is of public health importance, since it affects humans and dogs (DÁVILA & SILVA, 2000).

Among the species that affect cattle, *T.* (*D.*) *vivax* is the most important from a pathogenic perspective. For example, in Africa this protozoon is of great importance in relation to livestock. Wild ungulates act as reservoirs, and flies of the genus *Glossina* act as vectors (BOWMAN, 2010). The adaptive capacity of this parasite has allowed it to acquire the ability to be transmitted mechanically by hematophagous dipterans (e.g. *Tabanus* spp., *Stomoxys calcitrans* and *Haematobia irritans*), which have caused the spread of trypanosomiasis to Central America, South America and the Caribbean (SILVA et al., 2003).

In Brazil, occurrences of *T.* (*D.*) *vivax* were reported for the first time in buffaloes in the state of Pará (SHAW & LAINSON, 1972) and, over the last few years, it has been reported in other Brazilian states, such as Mato Grosso do Sul (PAIVA et al., 2000), Tocantins (LINHARES et al., 2006), Paraíba (BATISTA et al., 2007), Maranhão (GUERRA et al., 2008), Minas Gerais (CARVALHO et al., 2008; CUGLOVICI et al., 2010), Rio Grande do Sul (SILVA et al., 2009), São Paulo (CADIOLI et al., 2012), Pernambuco (PIMENTEL et al., 2012) and recently in Goiás (BASTOS et al., 2017). Because of the possibility of mechanical transmission by flies of the family Tabanidae, Muscidae and Hippoboscidae (RADOSTITS et al., 2007), as well as iatrogenic transmission through use of shared needles and syringes, this protozoon has disseminated rapidly throughout the country.

It is known that cattle infected by this protozoon may present acute, chronic or asymptomatic forms of the disease (BOWMAN, 2010). The main clinical signs of trypanosomiasis are progressive weight loss, pale mucous membranes, decreased milk production, anorexia, hyperthermia (RADOSTITS et al., 2007) and neurological signs (BATISTA et al., 2007; GALIZA et al., 2011). Infection by *T.* (*D.*) *vivax* may be diagnosed via parasitological methods, in which the characteristics of the general structure of the blood forms are observed using optical microscopy; or via serological methods, i.e. the immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (RADOSTITS et al., 2007); or via molecular methods. i.e. the polymerase chain reaction (PCR) (MADRUGA et al., 2006).

Considering the recent dispersion of *T.* (*D.*) *vivax* in Brazil and the economic impact that this protozoon may have, the aim of this study was to report and clinically, epidemiologically and molecularly characterize an outbreak of trypanosomiasis due to *T.* (*D.*) *vivax* in cattle in the state of Sergipe, northeastern Brazil.

Materials and Methods

Study area and sample collection

In August 2015, animals on a cattle farm in the municipality of São Miguel do Aleixo (10° 23' 26" S and 37° 22' 42" W), state of Sergipe, northeastern region of Brazil, presented with clinical signs relating to trypanosomiasis. Each animal was physically examined and clinical data reported in individual chart.

Blood samples were collected from the jugular vein of female lactating cattle (n = 15) and the material collected was placed in 4 mL sterile tubes with EDTA anticoagulant. After parasitological processing, the remained blood was stored at -20 °C until molecular analysis.

Laboratory analysis

Parasitological diagnosis

The parasitological diagnosis was made via blood smears on microscope slides and via the buffy coat method (MURRAY et al., 1977). The slides were stained using the rapid panoptic method (Laborclin) and were observed under an optical microscope (40X and 100X). The parasitic structures found were measured using the AxioVision software (release 4.8). All measures and morphological analyses were based on previous studies (JOHNSON, 1941; SHAW & LAINSON, 1972; OLIVEIRA et al., 2009).

Molecular diagnosis

Genomic DNA was extracted from 200 µl of bovine blood using a commercial kit (Qiagen DNeasy blood and tissue kit; Hilden, Germany) following the manufacturer's recommendations. PCR was performed in accordance with the procedure described by Geysen et al. (2003), using the primers 18STnF2 (5-CAACGATGACACCCATGAATTGGGGA-3) and 18STnR3 (5-TGCGCGACCAATAATTGCAATAC-3), which amplified a fragment of 659 bp of the 18SrRNA gene of *T.* (*D.*) *vivax.* DNA samples from the blood of a naturally infected cow (PIMENTEL et al., 2012) and ultrapure DNase free water (LGC®) were used as positive and negative controls, respectively. The reaction of amplification was performed in the following conditions: 1 cycle of initial denaturation at 94 °C for 4 minutes, followed by 40 denaturation cycles at 94 °C for 60 seconds, annealing at 58 °C for 90 seconds, extension at 72 °C for 120 seconds.

Randomly selected amplicons obtained from the positive samples were purified using the Qiaex II kit (Qiagen®; Hilden, Germany) and were sequenced in both directions using the Sanger method (SANGER et al., 1977) in an automated sequencer ABI-3130 (Applied Biosystems). The identity of the DNA sequences was determined by comparison with sequences available in GenBank, using BLASTn (ALTSCHUL et al., 1990). A phylogenetic tree was constructed using the UPGMA method (SNEATH & SOKAL, 1973). All sequences used in this study

for construction of the phylogenetic tree were available in the GenBank database. Bootstrap resampling (1000 replicates) was performed for statistical support regarding the reliabilities of the nodes on the trees (FELSENSTEIN, 1985) using the MEGA software, version 6.0 (TAMURA et al., 2013).

Data analysis

The statistical analysis to assess the parasitological methods was performed through the Fisher exact test using the BioEstat 5.0 software (AYRES et al., 2000).

Results

Out of the total of 15 lactating cows examined, 20% (3/15) presented hyperthermia, decreased milk production, anorexia, apathy, pale mucous membranes, tachycardia, tachypnea, dehydration and enophthalmos. Of these, 6.7% (1/15) presented neurological signs, including incoordination, muscle tremors, hypermetria and dysmetria. In addition, a high number of cases of abortion (33.34%; 5/15) was observed among the animals that were studied on this farm, along with a high number of deaths (20%; 3/15).

Table 1. Biometric mean and standard deviation (μm) of the number of specimens of *Trypanosoma* (*Duttonella*) *vivax* observed in blood smears from naturally infected cattle.

	X ± SD
Total lenght, including flagellum	22.55 ± 1.81
Distance from the kinetoplast to the posterior region	1.18 ± 0.38
Distance from the nucleus to the kinetoplast	4.76 ± 0.29
Distance from the posterior region to the nucleus	5.78 ± 0.30
Distance from the anterior region to the nucleus	8.08 ± 0.62
Free flagellum	7.61 ± 0.94

Through the laboratory analysis, the protozoon was detected in 34% (5/15) of the blood smears and 60% (9/15) of the buffy coat. There was no statistical difference between the parasitological methods (p = 0.0420). The protozoon was morphologically and morphometrically identified as *T.* (*D.*) *vivax* (Table 1).

In the molecular analysis on the bovine blood samples, 80% (12/15) were positive for a fragment (659 bp) with 99% similarity to *T. vivax* sequences available from GenBank (accession numbers KM391829, KM391827, KM391826, HM209400, KM391823 and AY362546). The sequence obtained in the present study was deposited in GenBank under accession number KX766453.

In the phylogenetic analysis, the isolates of *T.* (*D.*) *vivax* clustered separately from other *Trypanosoma* species (*T. congolense* and *T. evansi*). This was supported by a high bootstrap value (Figure 1).

Discussion

This study detected for the first time the infection due to *T.* (*D.*) *vivax* in dairy cattle in the state of Sergipe, Brazil. Although infection due to *T.* (*D.*) *vivax* does not present with specific clinical signs, some signs described here are compatible with those already reported in the scientific literature (LINHARES et al., 2006; FIDELIS et al., 2016). Interestingly, one animal presented neurological signs that were consistent with reports from an outbreak of *T.* (*D.*) *vivax* in cattle in the Brazilian semiarid region (BATISTA et al., 2007). There have also been reports of abortion on the farm evaluated here, similar to the findings of higher numbers of cases in the state of Pernambuco in cows infected by *T.* (*D.*) *vivax* (PIMENTEL et al., 2012). In addition, a mortality rate of 20% was observed here, among the positive animals.

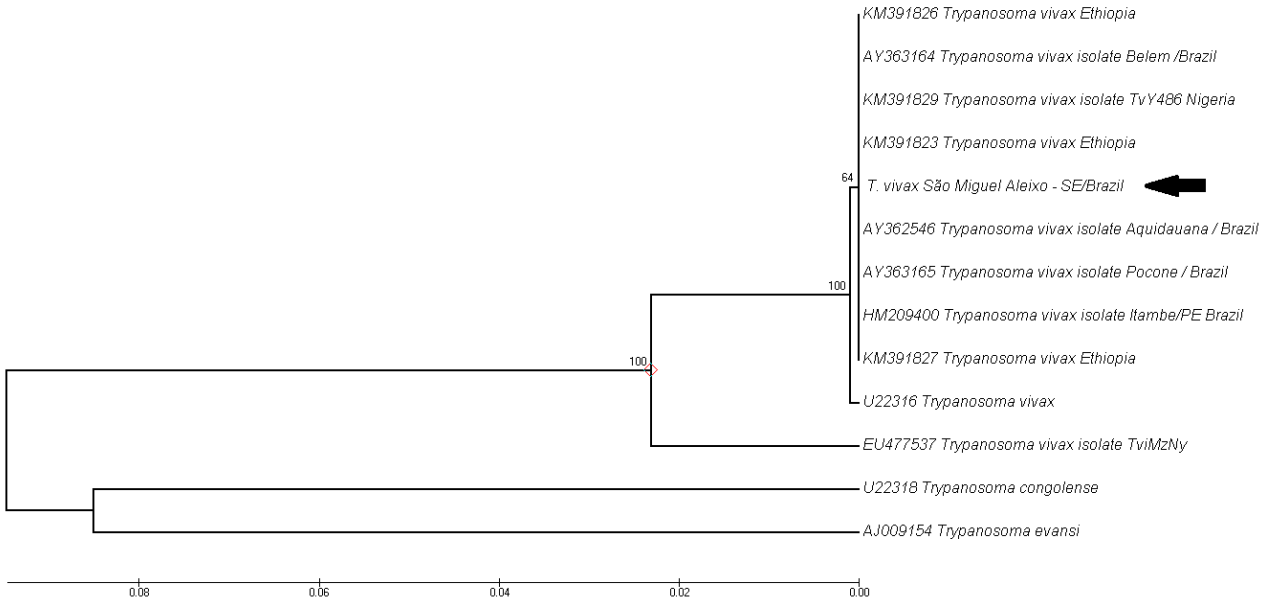

Figure 1. Phylogenetic tree based on *Trypanosoma vivax* 18SrRNA gene sequences. Sequences were compared using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar represents the number of mutations per sequence position.

The rate of positive parasitological diagnosis was lower than the rate observed via the molecular method. Although the parasitological method presents low sensitivity, it is known to be commonly used in several parts of the world to diagnose infections due to *T.* (*D.*) *vivax* (MADRUGA et al., 2006). However, parasitological techniques present low sensitivity in the chronic phase, whereas in the acute phase the sensitivity is higher depending on the parasitemia level (MADRUGA et al., 2006).

In the present study, the molecular diagnosis (PCR) on the samples analyzed showed a positivity rate of 80% (12/15) for *T.* (*D.*) *vivax*. This rate was lower than what was previously reported in the northeast region, in which 100% (22/22) of the animals were positive (PIMENTEL et al., 2012). The primers used in the present study amplify fragments of lengths of between 700 and 800 bp, depending on the species of *Trypanosoma* involved (GEYSEN at al., 2003). Using the same primers, Madruga et al. (2003) found that the fragment amplified was 659 bp in Brazilian samples of *T.* (*D.*) *vivax*. Therefore, the amplicons obtained in the present study were compatible in size to the ones described for Brazilian isolates of *T.* (*D.*) *vivax*. Thus, it can be concluded that PCR is a sensitive method for detection of *T.* (*D.*) *vivax,* which results in higher confidence in the diagnosis of trypanosomiasis.

In this report on *T.* (*D.*) *vivax* on a farm in São Miguel do Aleixo, the histories of the cattle involved showed that the first cases occurred after animals had been purchased and introduced from farms that traded in animals from other states. This suggests that the protozoon was introduced through acquisition of animals without previous knowledge of their health status. Another important factor is that on the farm studied here, oxytocin was administered intravenously to the cows every day before milking, using the same needle and syringe for all animals. Therefore, iatrogenic transmission seems to be the most important factor regarding the spread of *T.* (*D.*) *vivax* among these animals. After confirmation of the diagnosis of infection due to *T.* (*D.*) *vivax,* the farmer's attention was drawn to this, to avoid this form of transmission, especially regarding the need to avoid sharing of needles between animals.

In conclusion, presence of *T.* (*D.*) *vivax* in cattle herds is problem for livestock producers. Diagnosing trypanosomiasis is problematic because its evolution is similar to that of other parasitic blood diseases. Therefore, close attention to transit of animals from regions where the parasite is present is needed, along with care and clarification regarding the risks of sharing needles, in order to avoid economic losses associated with infection caused by this protozoon.

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