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Research Article

Serological and Parasitological Study of Bovine Trypanosomiasis in the Microregion of Uberaba, Minas Gerais State, Brazil

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Abstract

The microregion of Uberaba, in Minas Gerais state, Brazil, is an important center for breeding, genetic development, and cattle related business. Since 2011 outbreaks of bovine trypanosomiasis have been noticed in the region, leading to high mortality of bovines. Within this context, this study aimed to perform serological and parasitological screening of trypanosomiasis in the microregion of Uberaba. Blood and serum samples of 308 animals from the microregion of Uberaba were collected for serological and parasitological analysis. Data concerning the type of activity performed on each property and on the management of needles were also obtained in order to correlate with the presence of *Trypanosoma vivax*. The parasitological study was performed using Woo, Buffy Coat and Polymerase Chain Reaction (PCR) techniques; 100% of the samples were negative for Woo e Buffy Co at techniques, and 1.94% positive for PCR technique. The serological study, performed by Indirect Immunofluorescence (IIF) Reaction, a prevalence of anti *T. vivax* antibodies was recorded in 17.2% of the samples. The highest prevalence was found in the city of Uberaba, with 24.4%, followed by Veríssimo, with 14.3%; the other cities had prevalence lower than 9%. The prevalence was higher in dairy farms (75%) and in farms where needles were not exchanged, especially after oxytocin application in cows previous to milking (80%). The prevalence of trypanosomiasis in Uberaba-MG is low, and prophylactic measures must be implemented in order to prevent the spread of the disease.

ABBREVIATIONS

T. Vivax: *Trypanosoma Vivax*; EDTA: Ethylenediaminetetraacetic Acid; DNTP: Deoxynucleotide; IIF: Indirect Immunofluorescence; PCR: Polymerase Chain Reaction; PBS: Phosphate Buffered Saline; CEEA: Ethics Committee on Animal Experimentation

INTRODUCTION

Bovine trypanosomiasis, caused by *Trypanosoma vivax*, is wide spread in the African continent, where it has major socioeconomic consequences, particularly in tsetse-infested areas (genus *Glossina* spp) [1,2]. *Glossina* spp is the only vector in which *T. vivax* is able to multiply and remain infective throughout the life of the insect [3]. However, its adaptation to transmission by blood-sucking insects such as *Tabanus* spp. and *Stomoxys* spp. allowed the mechanical transmission to vertebrate hosts, which then led to the spread of *T. vivax* outside the African tsetse area to Central America, South America and Caribbean [3-5], which was probably caused by cattle exportation from Africa to other countries in the late nineteenth century [6]. The disease can cause acute and chronic infections in ruminants, leading to severe hematological disorders, loss of body condition, and decrease in productivity, eventually leading to animal death [3,7].

The main diagnostic methods for *T. vivax* are in parasitological analysis blood smear test, microhematocrit centrifugation technique, also called Woo test [8] and Buffy Coat technique. In serological analysis, the diagnostic consists in detection of antibodies anti-*T. vivax* and is performed using indirect immunofluorescence assay. In addition, the DNA detection is performed using molecular analysis by polymerase chain reaction technique [9].

The occurrence of *T. vivax* in Brazil was first reported in buffaloes in the state of Pará in the early 1970`s [4], and it

later disseminated to several regions of the country [10]. In Southeastern Brazil, specifically in the state of Minas Gerais, *T. vivax* was first reported in a cow in Igarapécity in 2007 [11]. Considering that the state of Minas Geraish as the highest milk production in Brazil, having a cattle herd estimated in of 22,69 million heads, 388,881 of them located in the micro region of Uberaba-MG which is an important axis in cattle breeding, genetic development and trading. Within this context, this study aimed to perform a serological and parasitological study of trypanosomiasis in the microregion of Uberaba, by correlating the presence of *T. vivax* with the type of rural activity and management strategies of the properties investigated.

MATERIALS AND METHODS

The study was conducted in the microregion of Uberaba, which includes the municipalities of Uberaba, Conceição das Alagoas, Conquista, Campo Florido and Veríssimo in the state of Minas Gerais, Brazil. This region is located at 19° 45' 27" south latitude and 47° 55' 36" west longitude, with a maximum altitude of 1,031m and a minimum altitude of 522 meters over a total area of 4,540.51 square kilometers, of which only 256 square kilometers are located in an urban area. According to the Köppen Climate Classification System, the climate in this region is tropical wet and dry, with tropical cold and dry winter (15/16°C). Average annual rainfall and temperature are 1,474 millimeters and 22.6°C, respectively.

Blood samples of 308 crossbred cattle, healthy, female, over 24 months of age, from 34 farms of the microregion of Uberaba-MG were collected by jugular venepuncture into tubes containing EDTA as anticoagulant for parasitological diagnosis by capillary tube centrifugation technique [8], blood smear slides [12], and into tubes without anticoagulant for serological and indirect immunofluorescence [13]. The number of samples was determined in accordance with the methodological guidelines of the Pan American Zoonosis Center [14].

Epidemiological data was obtained using questionnaire to the farm owners, aiming to obtain information regarding the properties such as kind of farm activity (Beef cattle, dairy cattle or mixed) and utilization of needle (if it is or not changed between one animal and another, if it is disinfected after usage).

The observation of blood trypomastigotes was conducted according to the literature. For each blood sample, two micro hematocrit tubes were filled in 2/3 of its total volume. Then, the rear end of the micro-tube was sealed with flame and centrifuged at 12,000 rpm for 5 minutes. The trypomastigotes forms were observed using optical microscope (Nikon Eclipse 2000®) (40x) in the portion between the leukocyte coat and plasma [8]. Posteriorly, the micro hematocrit tubes were broken between the leukocyte coat and initial portion of red blood cells. The smear was made using the portion containing plasma and leukocyte and dyed using quick Panoptic kit and examined under an optical microscope with objective Immersion (1000 times magnificence), according methodology [12].

DNA was extracted from blood samples using QIA amp DNA Mini Kit® (reference number 51306, Qiagen®), according to the manufacturer's instructions. *T. Vivax* specific PCR was performed according to protocol described in the literature [14], using the Tvi SL1 primer (forward: 5´GCTCTCCCAATCTTAACCCTA3´) and the TviSL2 primer (reverse: 5´GTTCCAGGCGTGCAAACGTC3´), aiming to amplify a fragment of approximately 210 base pair. The amplifications were performed in a mixture containing 3µL dNTPs, 6µL of both Tv SL1 and Tv SL2 primers (3µL each), 2µL of sample DNA, and PCR buffer containing 3µL of $MgCl_2$ and 0.2µL of Taq DNA polymerase. The reactions were carried out in MyCycler thermocycler (Bio-Rad®), with a total capacity of 96 tubes, which was adjusted to 35 cycles. Each cycle consisted of denaturation at 94ºC for 1 minute, annealing at 52ºC for 2 minutes, and extension at 72°C for 3 minutes, followed by a 10-minute final extension at 72°C.Two controls, one negative (negative bovine blood in IIF) and one positive (positive experimentally inoculated blood sample from sheep) were added to the reaction for comparison with the samples of this study. After the amplification, the products were submitted to electrophoresis in agarose gel 1% in 80Volts for 2.5 hours; 10µg/mL Ethidium Bromide was added, and gel reading was performed in ultraviolet transilluminator. The result of the amplified sections of both positive and negative controls was compared with DNA molecular weight markers (1kb DNA Ladder/Fermentas® and 100kb DNA Ladder/Fermentas®).

Indirect immunofluorescence reaction was performed using slides containing trypomastigotes of *T. vivax*, according described methodology [13]. Fluorescence reactions with titers ≥ 1:80 were considered positive [15].

After the experiments, the data were tabulated and submitted to statistical analysis by the chi-square test, with a significance level of 5% ($p < 0.05$). The strength of association was measured by Odds Ratio with a 95% confidence interval.

RESULTS AND DISCUSSION

The type of activity performed in 34 properties in the microregion of Uberaba-MG, as well as the procedure adopted in each property regarding needles are described in Table (1).

During the experiment, none of the 308 samples analyzed by Woo test and Buffy Coat test had *T. vivax* trypomastigotes. The samples evaluated by PCR showed amplification of the Tvi SL genomic region in 1.94% (6/308), being five samples from Uberaba and one sample from Veríssimo region, all serologically positive. Considering the cutoff point to be 80 in indirect immunofluorescence reaction, 82,8% (255/308) were negative whereas 17.2% (53/308) were positive, that is, they had anti-*T. vivax* antibodies and among them, 5,66% (03) showed anti-*T. vivax* antibodies to the dilution of 1:160 (Figure 1).

Of the 53 animals positive for titer 80, there was a prevalence of 24,4% (40/164) in the municipality of Uberaba, 3,3% (1/30) in Conceição das Alagoas, 8,8% (3/34) in Conquista, 8.8% (4/45) in Campo Florido and 14.3% (5/35) in Veríssimo (Figure 2).

Among the properties that had positive animals, 60% (12/20) were located in Uberaba, 5% (1/20) in Conceição das Alagoas, 10% (2/20) in Conquista, 15% (3/20) in Campo Florido and 10% (2/20) in Veríssimo (Figure 3).

Regarding the type of activity performed in the positive properties, 66, 66% (8/12) of the farms in Uberaba consisted of dairy farming, 16.7% (2/12) had beef farming, and 16.7% (2/12) had mixed farming. In Conceição das Alagoas, Veríssimo and in

Table 1: Type of activity and needle procedure in the municipalities of the microregion of Uberaba-MG.

Municipalities	Type of Activity			Needle procedure			
	Beef	Dairy	Mixed	DC	C	C10	D
Uberaba	8	8	2	14	1	2	1
	(44,4%)	(44,4%)	(11,2%)	(77,8%)	(5,5%)	(11,2%)	(5,5%)
Conceição das Alagoas	01 (20%)	04 (80%)	-	03 (60%)	-	02 (40%)	-
Conquista	01 (33,3%)	2	-	1	-	1	1
		(66,7%)		(33,3%)		(33,3%)	(33,3%)
Campo Florido	-	03 (75%)	01 (25%)	03 (75%)	-	01 (25%)	-
Veríssimo	01 (25%)	03 (75%)	-	03 (75%)	01 (25%)	-	-

Legend: DC: Doesn't change needles; C: Changes needles; C10: Changes needles every ten animals; D: Disinfects needles.

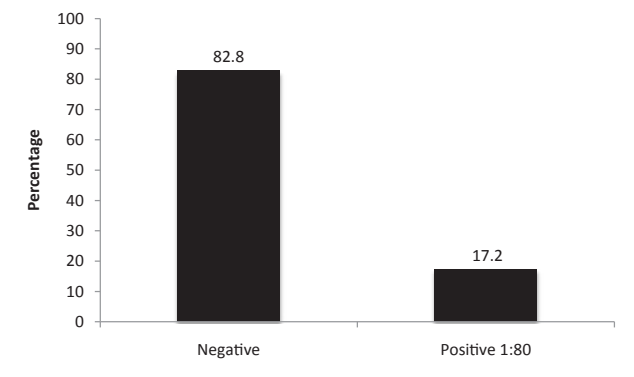

Figure 1 Negativity and positivity profile of cows in the microregion of Uberaba-MG.

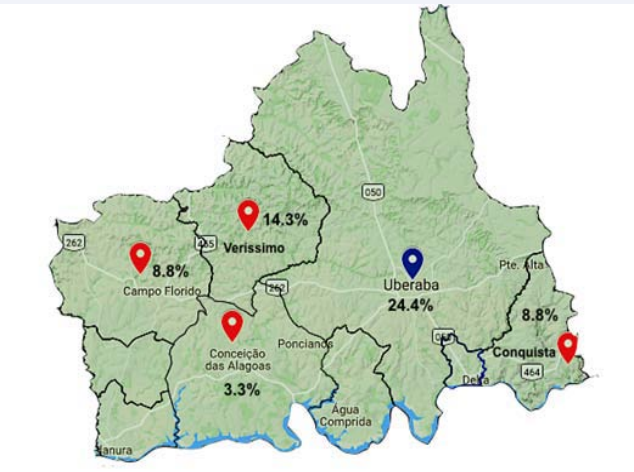

Figure 2 Prevalence of bovine trypanosomiasis in the municipalities of the micro region of Uberaba-MG.

Conquista, 100% of the farms owned dairy farming businesses (5/20, 4/20 and 3/20 respectively). In Campo Florido, 66.6% (2/3) properties had dairy farming and 33.4% (1/3) had mixed farming (Figure 4). The prevalence of anti-*T. vivax* antibodies in the animals located in farms raising dairy cattle in the municipalities of Uberaba microregion was statistically higher than the prevalence in farms that developed beef farming and mixed farming ($p < 0.05$).

In Uberaba, 91.6% positive properties (11/12) did not change needles between animals during vaccinations or administration of drugs, and 8.4% (1/12) changed the needles every 10 animals. The only positive property in Conceição das Alagoas changed the needles every 10 animals. In Conquista, 50% of the properties (1/2) changed the needles every 10 animals, and 50% (1/2) performed disinfection. In Campo Florido and inVeríssimo, 100% of the positive properties did not change needles (Figure 5). A statistically significant difference was observed in the positive properties that did not performed needle change ($p < 0.05$) (Table 2).

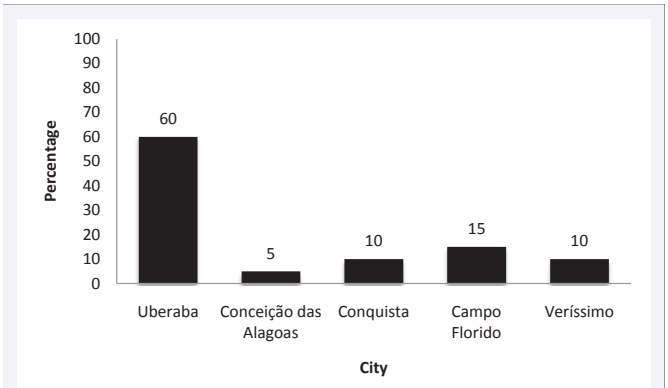

Figure 3 Percentage of the positive properties for T. vivax in the municipalities of the microregion of Uberaba-MG.

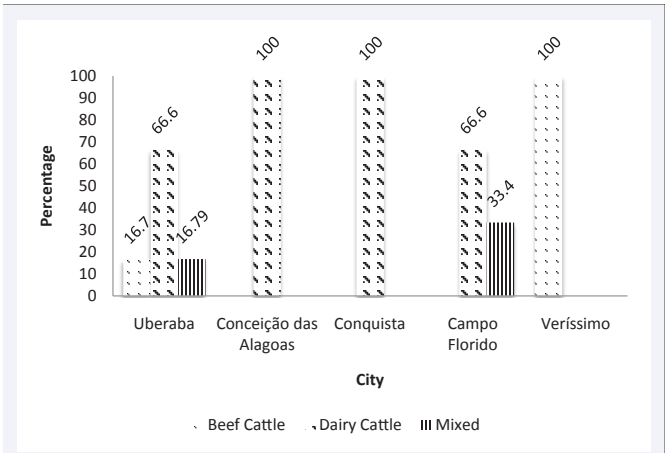

Figure 4 Profile of the properties positive for T. vivax regarding the type of activity developed.

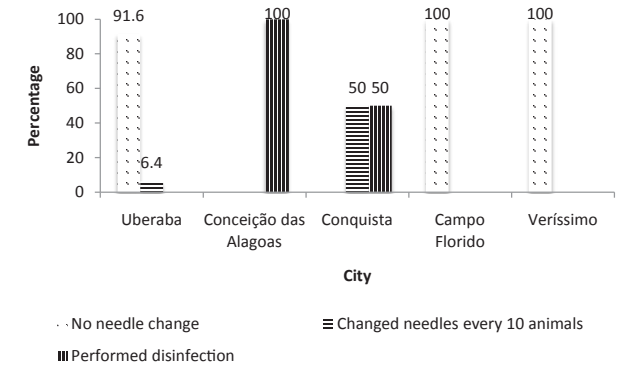

Figure 5 Profile of the properties positive for T. vivax regarding needle procedure.

Table 2: Odds ratio for activity and needle management in the different properties of the study.

Parameter	OR (IC95%)	Valor-p
Activity		
BeefCattle	1,00	< 0,001
DairyCattle	9,18 (3,21-26,27)	
Mixed	2,59 (0,55-12,19)	
Needle management		
No changing	7,42 (0,44-126,44)	0,157
Disinfects	1,88 (0,06-60,08)	
Changesevery 10 animals	2,61 (0,13-83,59)	
Changes	1,00	

Trypomastigotes of *T. vivax* were not detected in parasitological analysis of the 308 samples, which may be explained by the low sensitivity of the parasitological tests [13,16,17] and by absence of clinical disease in the animals (healthy animals).

The absence of parasites does not indicate that the animals are negative, because parasitemia is not detectable by blood smear or Woo test in the chronic phase of the disease [18].

The low prevalence observed in PCR in comparison with the serological test can also be explained by the low parasitemia in the chronic phase of the infection, which may lead to false-negative results by PCR [19].

Serological diagnostic methods such as IIF can show if the animals that were negative in parasitological tests were really negative or if they were in the chronic or subclinical phase of the disease. The chronic phase of the disease is associated with low and fluctuating parasitemia, and serological study is important to complement the parasitological examinations, since the animals can control parasitemia after exposure to the parasite [13]. This can be related to the responsiveness of the immune system of some animals (inborn or acquired resistance) or even to the low virulence of *T. vivax* isolates [20]. Of the 308 animals, 17.2% had anti-*T. vivax* antibodies (titer 80), so they were in the chronic or subclinical phase of the disease.

Prevalence rates higher than those of the epidemiological study carried out in the microregion of Uberaba were found in the northern state of Pará (83-96.7%) [10], in the state of Paraíba (49%; 41.6%) [2,21], and in Pernambuco [22], where was reported the first occurrence of *T. vivax* in 100% of the dairy herd in 2010. High prevalence rates in northern Brazil were also found, particularly in the subregion of the Pantanal (34.48%) [23]. The prevalence of the disease in the microregion of Uberaba is still noticeably lower than that in previous studies performed in other Brazilian states. Nonetheless, as dairy cattle breeding prevails in Uberaba region, where the movement of animals is often due to large cattle shows and which lacks proper management, there may be an increase in *T. vivax* cases in the region, thus causing social-economic loss, as suggested by authors [24].

The tendency to a increased prevalence in Uberaba is explained by the high genetic value cattle in the municipality, and by high animal movement from various states, especially during the livestock shows held throughout the year.

Animal movement allows the entry of animals infected with *T. vivax* and, hence, the onset of the disease. This can be seen in epidemiological studies in which the authors propose that the movement of animals was the probable cause of the introduction of this parasite in the state of Pernambuco [21,22];Were similar reasons proposed in regarding an outbreak Reported in Igarapé-MG [11].

According to Madruga [25], as animals in the chronic phase of the disease do not show any clinical signs, it is difficult to control the movement of animals, which may explain the appearance of the disease and the spread of the parasite.

In addition to the movement of cattle and to the holding of livestock shows in Brazil, another important point in the transmission of *T. vivax* is the type of activity carried out on the farms because dairy animals have an increased susceptibility to disease when they are gathered in corrals, which hold the largest population of flies [26]. When studying the *T. vivax* outbreaks in Paraíba state, Brazil, the autors attributed the presence of mechanical vectors, such as *tabanids* and *Stomoxys* spp, as one of the main factors responsible for the occurrence of this disease [2,21].

Upon correlating these epidemiological factors with the antibody titers found in the municipalities, antibodies were found to be more prevalent in the municipality with the greatest movement of cattle (Uberaba-MG, 24.4%), followed by municipalities where dairy farming was higher than 50% - that is, Veríssimo (75%), Campo Florido (75%), Conceição das Alagoas (80%) and Conquista (66.7%), and by properties developing only dairy farming.

Iatrogenic transmission is another important point to be analyzed as it directly affects the transmission of *T. vivax* trypomastigotes [27,28]. The fact that needles are not properly changed can also be considered a potential disseminator of disease, since the habit of not replacing the needle was found to be frequent in the properties studied. Moreover, reusing the same needle in several animals for oxytocin application pre-milking is a major factor in the transmission of *T. vivax* because the blood forms of the trypanosomes are directly transferred from one animal to another through needles contaminated with infected

blood. More than 80% of the properties of the municipalities of Uberaba, Veríssimo and Campo Florido did not perform needle replacement [9].

The high sero negativity percentage (82.8%) shows that animals in the microregion of Uberaba are susceptible to acquiring the disease, since they do not have anti-*T. vivax* antibodies, and if subjected to situations that facilitate the transmission of the agent, such as the use of contaminated needles, there may be intense presence of insect vectors, associated with stressful situations or immunosuppression situations, disease outbreaks, as well as occurrence of new cases, thus leading to high morbidity and mortality of the animals [24,29].

CONCLUSION

According to the results, it can be concluded that there is a low parasitological (1.94%) and serological (17.2%) prevalence of trypanosomiasis in the microregion of Uberaba-MG, Brazil, and that trypanosomiasis occurs mainly in dairy farms that do not dispose of the needles. These are important factors in the epidemiology of trypanosomiasis, and prophylactic measures should be implemented in the region so as to prevent the spread of the disease.

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