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SEROPREVALENCE OF *Trypanosoma vivax, Anaplasma marginale*, and *Babesia bovis* IN DAIRY CATTLE

SOROPREVALÊNCIA DE Trypanosoma vivax, Anaplasma marginale e Babesia bovis EM REBANHOS LEITEIROS

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Abstract

In order to determine the prevalence of IgG against *Trypanosoma vivax, Anaplasma marginale*, and *Babesia bovis* in dairy cattle in southern Minas Gerais, four hundred cows from 40 dairy farms were randomly selected and distributed in 14 municipalities. Seroprevalence was determined by indirect immunofluorescence assay (IFA). Interviews were conducted to characterize producers and dairy production. Univariate analysis was carried out using chi-square (x^2) or Fisher's exact test. The multiple model was constructed with variables associated with $p \leq 0.25$ by x^2 test using generalized estimating equations (GEE). True prevalence at herd level was 49.6% (31.7–67.5), 100% (92.1–100), and 100% (86.5–100) for *T. vivax, A. marginale*, and *B. bovis*, respectively. At individual level, true seroprevalence was 9.9% (6.7–13.1), 96.2% (92.1–99.6), and 93.7% (89.4–97.2), respectively, for *T. vivax, A. marginale,* and *B. bovis*. Among the factors adjusted by logistic regression GEE model, "total farm area" (p= 0.021, OR= 0.308, $Ic_{95\%}$= 0.114–0.836) and "fly season" (p= 0.016, OR= 2.133, $Ic_{95\%}$= 1.153–3.948) remained associated with seropositivity for *T. vivax*. As the State of Minas Gerais is considered non-endemic for *T. vivax*, producers and veterinarians should be informed about the risk of occurrence of bovine trypanosomiasis.

Keywords: anaplasmosis; babesiosis; dairy cattle; enzootic stability; trypanosomiasis.

Resumo

Com o objetivo de determinar a prevalência de anticorpos IgG anti-*Trypanosoma vivax, Anaplasma marginale* e *Babesia bovis* em bovinos leiteiros no Sul de Minas Gerais, quatrocentas fêmeas bovinas adultas provenientes de 40 propriedades leiteiras foram selecionadas aleatoriamente e distribuídas por 14 municípios localizados na região Sul de Minas Gerais. A soroprevalência foi determinada pela reação de imunofluorescência indireta (RIFI). Foram realizadas entrevistas para caracterizar os produtores e a produção leiteira. As análises estatísticas foram realizadas no software PASW 18. Foi realizada análise univariada pelo Qui-quadrado (x^2) ou Teste Exato de Fisher. Com as variáveis associadas com $p \leq 0,25$ pelo teste x^2 construiu-se o modelo múltiplo por meio de Equações de Estimação Generalizadas (GEE). A soroprevalência verdadeira em nível de rebanho foi 49,6% (31,7–67,5), 100% (92,1–100) e 100% (86,5–100) para *T. vivax, A. marginale* e *B. bovis*, respectivamente. Em nível individual, a soroprevalência verdadeira foi 9,9% (6,7–13,1), 96,2% (92,1–99,6) e 93,7% (89,4–97,2), respectivamente, para *T. vivax, A. marginale* e *B. bovis*. Dentre os fatores ajustados

pelo modelo de regressão logística GEE, as variáveis "área total da fazenda" (p= 0,021; OR= 0,308; IC95%= 0,114 – 0,836) e "época com maior número de moscas" (p= 0,016; OR= 2,133; IC95%= 1,153 – 3,948) se mantiveram associadas à soropositividade para *T. vivax*. Devido ao fato de o Estado de Minas Gerais ser considerado área não endêmica para a *T. vivax*, é importante que produtores e veterinários sejam informados quanto ao risco de ocorrência da tripanossomíase bovina.
Palavras-chave: anaplasmose; babesiose; bovinos leiteiros; estabilidade enzoótica; tripanossomíase.

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Introduction

Trypanosoma vivax (Kinetoplastida: Trypanosomatidae) causes bovine trypanosomiasis, a parasitic disease of great economic importance in Africa, whose biological vector is the tsetse fly. However, outside the African continent *T. vivax* underwent adaptation to mechanical transmission by blood-sucking flies such as horseflies (tabanids) and *Stomoxys calcitrans*, allowing for territorial expansion of bovine trypanosomiasis to several countries in Central America, South America, and the Caribbean[1]. Other transmission method is iatrogenesis via needles and instruments during vaccinations and mass treatments[2,3].

In Brazil, bovine trypanosomiasis caused by *T. vivax* was first reported in 1972 in buffaloes in the northern state of Pará[4]. For about two decades, this parasite was restricted to the North region, also occurring in the state of Amapá[5]. However, further outbreaks of bovine trypanosomiasis caused by *T. vivax* were confirmed in the North[6], Northeast[7,8], Midwest[9,10], Southeast[11,12], and South[13].

In endemic areas such as the Pantanal and North region, *T. vivax* rarely causes a clinical disease in cattle. However, when cattle infected with *T. vivax* from endemic areas are introduced in parasite-free areas, the agent can spread and cause outbreaks of trypanosomiasis in cattle, with severe clinical signs of disease and eventually death[7,14]. Severe anemia was the main sign of disease[15] in the first outbreak of trypanosomiasis caused by *T. vivax* in Minas Gerais, reported on a dairy farm in Igarapé[11]. An outbreak of *T. vivax* in cattle was also reported in Uberaba, in Minas Gerais in 2012, with 50% of positivity in 16 animals sampled during the outbreak. Added to this report, a serological study was developed in Uberaba, revealing a prevalence of *T. vivax* around 16.2% in 327 animals sampled[16].

In Brazil, *Babesia bovis* and *B. bigemina* (Piroplasmorida: Babesiidae) protozoa are the etiological agents of bovine babesiosis, while anaplasmosis is caused by obligate intraerythrocytic bacteria *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). The *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) tick is the main vector of these blood parasites that cause anemia in cattle from South and Central America[17], which are responsible for significant economic losses in dairy cattle in tropical and subtropical areas in the world[18].

Brazil is considered enzootic for *B. bovis* and *A. marginale*, with herd infection rates of 80–100% in several regions[19]. Enzootic instability situations are not common in the country and are usually associated with climatic factors causing seasonal variations in the population dynamics of vector tick *R. (B.) microplus*[20].

The state of Minas Gerais is a major hub of agricultural production and dairy farming in Brazil, accounting for approximately 25% (13,636 million head) of cows milked and 28% (20,156 billion liters) of total milk production in the country in 2010[21]. Southern Minas Gerais stands as a major dairy region, supplying big cities like São Paulo and providing raw material for the state's own dairy industries.

This study determined the prevalence of IgG antibodies against *T. vivax*, *A. marginale*, and *B. bovis* in dairy cattle in southern Minas Gerais. The state is considered non-endemic for bovine trypanosomiasis,

with a single report of disease occurrence so far. Considering the prominent role of the state in milk production nationwide and total lack of research on the prevalence of *T. vivax* in dairy cattle in the region, this study is epidemiologically relevant to monitor the serological status of the hemoprotozoan parasite in the region. The study is also clinically important to help veterinarians in differential diagnosis of cattle suffering from acute hemolytic syndrome, since bovine trypanosomiasis clinical signs are similar to those of bovine babesiosis and anaplasmosis.

Material and Methods

This observational cross-section study was conducted to evaluate the prevalence of IgG antibodies against *T. vivax, A. marginale*, and *B. bovis* in 400 apparently normal cows from 40 dairy farms, randomly selected and distributed in 14 municipalities in Southern Minas Gerais (45 W meridian, 21 S parallel) (Figure 1).

To calculate herd true prevalence, sensitivity and specificity tests were adjusted from individual level to herd level using Herdacc software[22]. A herd was deemed positive if at least one animal was tested positive. To calculate true prevalence of specific IgG antibodies against *T. vivax* we used values of sensitivity (85.7%), according to Ashkar and Ochilo[23], and specificity (100%) according to Platt and Adams[24]. Values of sensitivity (92%) and specificity (95%) for *B. bovis* were used according to Ogunremi et al.[25], and sensitivity (90.7%) and specificity (100%) for *A. marginale* according to Ekici and Sevinc[26]. Subsequently, sensitivity and specificity values among herds were used to calculate true prevalence at herd level[27], using Epitools epidemiological calculators[28]. The confidence intervals for true prevalence were calculated according to Reiczigel et al.[29].

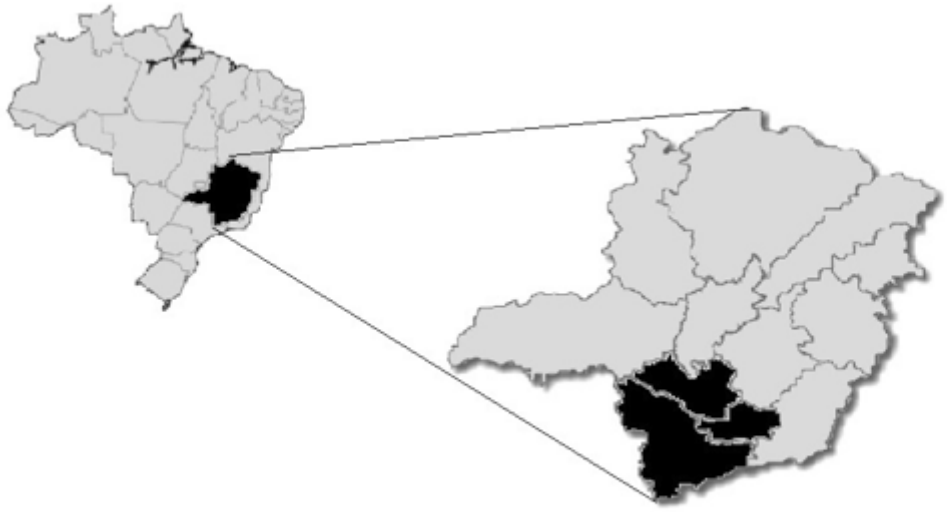

Figure 1. Map highlighting the three sampling areas (dark) in Southern Minas Gerais, Brazil.

Serum samples were drawn (10 sera per herd) from 1200 samples (30 sera per herd) used in a previous study that determined prevalence of IgG antibodies against *Neospora caninum* in dairy cattle in the sampling areas shown in Figure 1[30]. The number of animals (n) needed to estimate prevalence was based on the Pan American Zoonoses Center[31] formula: $n= [p \times (100-p) \times Z^2]/ (d \times p/100)^2$. Considering an estimated prevalence (p) of 50%, confidence level (z) at 95% of 1.96 and allowable margin of error (d) 10%, we obtained a minimum number (n) of 384 serum samples. Upon visiting the farms we conducted pre-tested semi-structured interviews to collect information (independent variables) on possible factors associated with occurrence of *T. vivax* and to characterize farmers and milk production. This interview form was approved by the Ethics Committee in the Use of Animals/ CEUA from Universidade Federal de Lavras under the protocol 082/2011. Information about herds is shown in Table 1.

Table 1. Dichotomized categories of each independent variable in statistical analysis associated with seropositivity for *Trypanosoma vivax* in dairy herds in Southern Minas Gerais, Brazil

N°	Characteristics	Code 0	Code 1
1	Total farm area *	≤110 ha	>110 ha
2	Wetlands	Yes	No
3	Production of type A milk	Yes	No
4	Production of type B milk	Yes	No
5	Production of type C milk	Yes	No
6	Activity type	Milk production	Mixed farming
7	Abortion occurrence	Yes	No
8	Bordering dairy farms	Yes	No
9	Veterinary care	Yes	No
10	Purchase of breeding cattle	Yes	No
11	Purchase of cattle from São Paulo	Yes	No
12	Purchase of cattle from Mato Grosso do Sul	Yes	No
13	Rented pasture	Yes	No
14	Shared pasture	Yes	No
15	Forestland	Yes	No
16	Livestock access to forestland	Yes	No
17	Frequency of reproductive problems *	≤ 20%	> 20%
18	Occurrence of rabies	Yes	No
19	Fly season*	Rainfall	All year round
20	Fly frequency*	Never, rarely, low	Medium, high
21	Frequency of horse flies (tabanids)*	Never, rarely, low	Medium, high

In this study, farms had a total median area of 110 hectares (interquartile range (IR) = 223) with exclusive area for milk production (corral, milking parlor, and pasture) of 20 ha (IR= 68). The median number of dairy cows was 82 (lactation + dry period) (Q1= 50.5; Q3= 148.75) and 62 lactating cows (Q1= 40; Q3 = 120). Average milk yield was 20 ± 7.4 liters per cow and 1,350 liters per day (Q1= 562.5, Q3= 2,287.5), indicating a high production well above the average of dairy farms in Brazil (41 liters/day) and particularly in the State of Minas Gerais (69 liters/day)[32].

Seroprevalence was determined by indirect immunofluorescence assay (IFA) according to the

technique described by the Inter-American Institute on Cooperation for Agriculture[33]. Antigens were trypomastigotes of *T. vivax* from splenectomized goats experimentally infected with strain Igarapé/MG[15] and antigens of *A. marginale* and *B. bovis* prepared as described by Carvalho et al.[34]. After being thawed at room temperature, the sera were tested for *T. vivax* without PBS dilution. Then, the sera considered reagent for IgG against *T. vivax* were retested and deemed positive if showing reaction at dilution 1:80[15]. Sera were considered positive for *B. bovis* and *A. marginale* if reacted at dilution 1:320[34]. In the preparation of slides for IFA we used serum test for each agent, positive and negative controls, and conjugated anti-bovine IgG (Sigma, St. Louis, MO, USA) diluted 1:100.

Statistical analyzes were performed using PASW 18 software. Before applying statistical tests, almost all questionnaire questions were classified, dichotomized, and evaluated based on response frequency. For example, single response variables were excluded.

Factors associated with seropositivity for *T. vivax* were identified by statistical analysis, considering IFA serological results as a dependent variable and questionnaire data as independent variables. Thus, the association between dependent and independent variables was initially assessed by chi-square univariate analysis (x^2) or Fisher's exact test (less than five observations in at least one cell in the contingency table). Odds ratio (OR) was calculated for the statistically related variables ($p < 0.05$) at confidence interval of 95%. The variables subjected to x^2 test were evaluated for collinearity to construct the multiple model. When variables correlated (coefficients> 0.8), the variable more likely to be related to seropositivity for *T. vivax* was maintained. When $p \leq 0.25$ by x^2 test, the variables were selected to construct the multiple model[35].

Association between significant variables at herd level and seroprevalence were assessed using generalized estimating equations (GEE). This model is more appropriate when the associated data are found in different dimensions, such as in this study assessing cows from different farms[36]. Each farm was considered a subject and each animal a case, assuming a relationship of dependency between cows raised on the same farm and independence between animals raised in different properties. The risk for all variables associated on the final multiple model ($p < 0.05$) was estimated using the adjusted OR at confidence interval 95%.

Results

In most farms, dairy farming is the only source of income (55.0%) while others produce refrigerated raw milk (52.5%) and raise crossbred cattle (51.3%). Furthermore, most farmers have been producing milk on the same farms for over 20 years (51.3%), and 65.0% of dairy cattle workers have only minimal education (4th grade elementary school). Nevertheless, in most farms (60.0%) cows were raised in a semi-intensive system and milked twice a day (87.5%) by automatic milking systems (72.5%). Artificial insemination (65.0%) was the main reproductive method.

True prevalence at herd level for *T. vivax*, *A. marginale*, and *B. bovis* was 49.6% (31.7–67.5), 100% (92.1–100), and 100% (86.5–100), respectively. At individual level, values were 9.9% (6.7–13.1), 96.2% (92.1–99.6), and 93.7% (89.4–97.2) for *T. vivax, A. marginale,* and *B. bovis*, respectively. Factors associated with seropositivity for *A. marginale* and *B. bovis* were not analyzed in this study because of the high true seroprevalence found for these hemoparasites at both herd and individual levels.

Eight variables were used in the multivariate analysis (Table 2), which considered data of 400 cattle from 40 herds. We found a significant association ($p < 0.05$) with seropositivity for *T. vivax* in the univariate analysis (x^2) for some factors related to farm structure and management, such as total farm area, occurrence of horse flies (tabanids), and flies. However, after adjustment by logistic regression GEE model, only the variables 'total farm area' (≤ 110 ha and > 110 ha) ($p = 0.021$, OR= 0.308, $Ic_{95\%}$= 0.114–0.836) and 'fly season' (all year round) ($p = 0.016$, OR = 2.133, $Ic_{95\%}$= 1.153–3.948) remained significantly associated ($p < 0.05$) with serum positivity for *T. vivax*.

Table 2. Variables with p ≤0.25 by chi-square test used in the construction of Generalized Estimating Equations (GEE)

Characteristics	Frequency 0	1	Missing	Total
Total area				
≤ 110 ha (0), > 110 ha (1)	20	19	1	40
Frequency of reproductive problems				
≤ 20% (0), > 20% (1)	28	11	1	40
Fly season				
Rainfall (0), all year round (1)	38	2	0	40
Type B milk				
No (0), Yes (1)	24	16	0	40
Occurrence of horse flies (tabanids)				
Never, rarely, low (0), Medium, high (1)	32	8	0	40
Type C milk				
No (0), Yes (1)	18	22	0	40
Type of purchase				
Calves and Heifers (0), Cows (1)	7	5	28	40
Occurrence of flies				
Never, rarely, low (0), Medium, high (1)	9	31	0	40

Discussion

Most of the Brazilian cattle farms are established in areas of enzootic stability for *A. marginale* and *B. bovis*, with seroprevalence rates of 80–100% in dairy farms[19]. In areas of enzootic stability there is a balance between immunity and disease, where 75% or more of cattle over nine months old are seropositive for hemoparasites[37]. In this study, true prevalence rates at farm level and animal level to *A. marginale* and *B. bovis* were higher than 75%, with a confidence interval reaching 100%. This result is consistent with those reported in other studies conducted in Brazil[19], and particularly in dairy cattle in Southern Minas Gerais[38], which is characterized as enzootic stable for *A. marginale* and *B. bovis* with low risk of anaplasmosis or acute babesiosis in native adult animals.

True prevalence rate of *T. vivax* at individual level was 9.9%, similar to the rate found in the first and only outbreak of trypanosomiasis by *T. vivax* reported in Minas Gerais so far (7.4%) on a dairy farm in Igarapé, metropolitan mesoregion of Belo Horizonte[15], and below the rate found in Pernambuco, 14%[8]. However, prevalence rates of 30.7–93.1% are not uncommon in Brazilian endemic areas such as Pantanal and North Region[9,39,40], well above those reported in Southern Minas Gerais.

We found a significant association ($p < 0.05$) between fly season and seroprevalence for *T. vivax* in dairy cattle. Occurrence of high amount of flies throughout the year increases probability of seropositivity for *T. vivax* compared to farms where a fly season occurs. According to Batista et al.[41], chronically infected animals and mechanical vectors (*S. calcitrans* and *Tabanus* spp.) contribute to the spread of *T. vivax* infection in cattle herds.

There was also a significant association ($p < 0.05$) between total farm area and seroprevalence for *T. vivax*. Farms larger than 110 hectares have lower chance of occurrence, probably due to a higher level of technology, appropriate health management, and constant veterinary care.

The results indicate that Southern Minas Gerais is an area of enzootic stability for *A. marginale* and

B. bovis, with low risk of blood parasite disease in adult native cattle. In this region, dairy cattle are exposed to *T. vivax*. No higher seroprevalence for *T. vivax* was found in cattle raised on farms that had purchased animals from areas of occurrence of *T. vivax*, such as the states of São Paulo, Mato Grosso, and Mato Grosso do Sul. However, according to Cadioli et al.[12], the source of contamination in the first outbreak of *T. vivax* in São Paulo was cattle from infected farms in Mato Grosso do Sul. Within six months, 100% of cows seroconverted on the farm where infected animals had been introduced.

Thus, probably the occurrence of dairy cattle infected with *T. vivax* in Southern Minas Gerais is due to the introduction of chronically infected animals from endemic regions in areas considered free of *T. vivax*, with blood-sucking flies (tabanids and *S. calcitrans*) acting as mechanical vectors. This argument is supported by studies conducted in Brazil, which first reported the occurrence of outbreaks of trypanosomiasis in areas considered free of *T. vivax*[11,12,15]. Also, goats can be asymptomatic reservoirs and an important source of *T. vivax* for cattle[42].

The high prevalence of *T. vivax* among dairy herds in Southern Minas Gerais indicates the need for health care to prevent spread to regions considered free of the parasite, within or outside the state. As cattle maintain a chronic silent infection, farmers should be advised to verify the origin of the livestock they purchase. Moreover, as acute bovine trypanosomiasis caused by *T. vivax* is severe, veterinarians should be prepared to undertake the diagnosis and early treatment. According to Batista et al.[14], however, proper diagnosis is complicated by similarity between clinical signs of trypanosomiasis and other diseases, added to the lack of knowledge of *T. vivax* infection. Diseases such as anaplasmosis and babesiosis, which have similar clinical signs to bovine trypanosomiasis (fever, anemia, and lethargy, loss of appetite, reduced production, and abortion), can hinder an accurate diagnosis[14,15,42]. Thus, veterinarians should be informed of the risk of bovine trypanosomiasis caused by *T. vivax* and clinical suspicion in Southern Minas Gerais, especially in cases of adult cattle with acute hemolytic syndrome and severe anemia.

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