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Prevalence of '*Candidatus Mycoplasma haemobos*' detected by PCR, in dairy cattle from Ji-Paraná in the north region of Brazil

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ABSTRACT: The municipality of Ji-Paraná, Rondônia, is one of the major dairy production areas in the north region of Brazil. Thus, it is important to evaluate infectious agents that have the potential to negatively affect productivity in the industry. The objective of this study was to investigate the prevalence of '*Candidatus Mycoplasma haemobos*' by using a PCR-based detection method and correlate this with dairy herd variables (abortion frequency, weak calf birth rate, total cattle number, >24-month-old cow number, farm size, and production system) in family farms of the Ji-Paraná municipality, north region, Brazil. Blood samples were collected from 320 dairy cows located across 64 farms (i.e., five animals per farm) from September 2012 to November 2013. Overall prevalence of '*Ca. M. haemobos*' was 64.2% and prevalence per herd was 95.3%; the number of >24-month-old cows in the farms studied correlated with '*Ca. M. haemobos*' infection rates. Considering the importance of the dairy industry to the study area, additional investigations are necessary to evaluate the effect of chronic infection in these animals on milk production and herd health.

Key words: hemoplasmas, hemotropic mycoplasmas, dairy herd.

Prevalência de '*Candidatus Mycoplasma haemobos*' pela PCR, em bovinos leiteiros de Ji-Paraná na região norte do Brasil

RESUMO: O município de Ji-Paraná, Rondônia, é uma das principais áreas de produção leiteira na região norte do Brasil. Assim, é importante avaliar agentes infecciosos que apresentam potencial para afetar negativamente a produtividade na indústria. O objetivo deste estudo foi investigar a prevalência de '*Candidatus Mycoplasma haemobos*' usando um método de detecção baseado na PCR e relacionar com variáveis do rebanho leiteiro (frequência de aborto, nascimento de bezerras fracas, número total de vacas, número de vacas >24 meses de idade, tamanho da propriedade e, sistema de produção) na agricultura familiar do município de Ji-Paraná, região norte, Brasil. Amostras de sangue foram coletadas de 320 vacas leiteiras localizadas em 64 fazendas (ou seja, cinco animais por exploração) de setembro de 2012 a novembro de 2013. A prevalência total de '*Ca. M. haemobos*' foi de 64,2% e a prevalência por rebanho foi de 95,3%; o número de vacas >24 meses de idade nas propriedades estudadas correlacionou-se com as taxas de infecção por '*Ca. M. haemobos*'. Considerando a importância da indústria de laticínios para a área de estudo, investigações adicionais são necessárias para avaliar o efeito da infecção crônica nestes animais na produção de leite e saúde do rebanho.

Palavras-chave: hemoplasmas, micoplasmas hemotrópicos, rebanho leiteiro.

INTRODUCTION

Hemotropic mycoplasmas, also known as hemoplasmas, are unculturable and cell wall-less bacteria whose infections cause anemia in several mammalian species by attaching erythrocytes (SMITH et al., 1990; MESSICK, 2004). Two distinct species have been identified in cattle, namely, *Mycoplasma wenyonii* (formerly *Eperythrozoon wenyonii*) (ADLER & ELLENBOGEN, 1934) and a provisional species '*Candidatus Mycoplasma haemobos*', also known as '*Ca. M. haemobovis*' (TAGAWA et al., 2008).

Despite the worldwide distribution of both bovine hemoplasmas, '*Ca. M. haemobos*' has only

been reported in cattle from Switzerland (MELI et al., 2010), China (SU et al., 2010), Japan (TAGAWA et al., 2008), Germany (HOELZLE et al., 2011), Hungary (HORNOK et al., 2011), England (AYLING et al., 2012), New Zealand (MCFADDEN et al., 2016), and Brazil (GIROTTI et al., 2012). The latter refers to the only description of hemoplasma in cattle blood detected by PCR in the southern region of the country. It is difficult to interpret the pathogenic potential of bovine hemoplasma infections since co infection may contribute to hemoplasma pathogenicity in a synergistic manner, particularly when infection results in immunosuppression (MELI et al., 2010; MCFADDEN et al., 2016). Chronic infection in cattle can be asymptomatic (SMITH et

al., 1990; MESSICK, 2004; TAGAWA et al., 2013); however, symptoms can be observed such as transient fever, anorexia lymphadenopathy, and weight loss in females and calves.

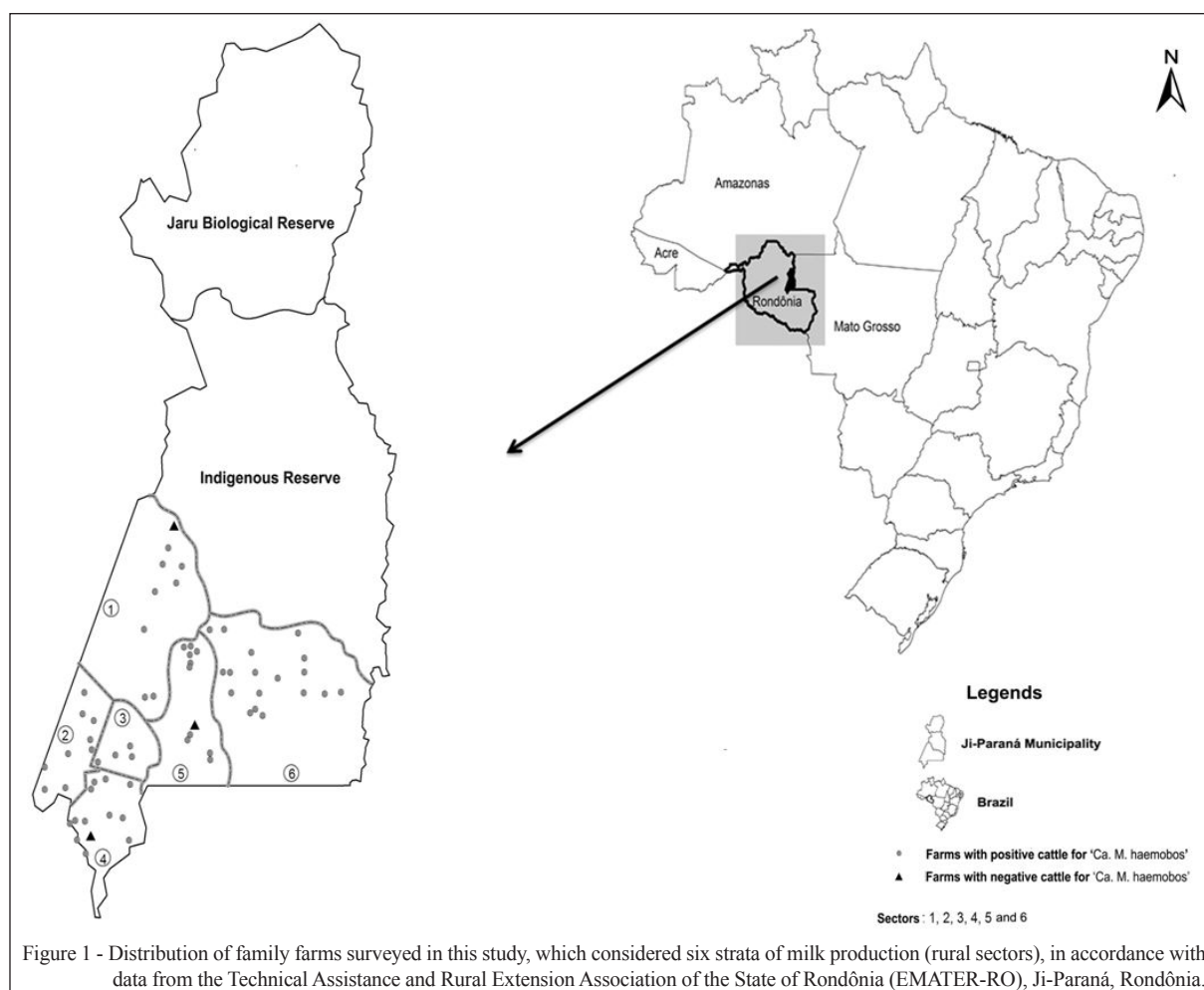
Decreased milk yield, abortion, and delayed estrus have been reported during the acute phase of hemoplasma infection in cattle (SMITH et al., 1990; MESSICK, 2004; TAGAWA et al., 2013). In addition, previous studies have observed the effect of hemoplasma infection on hematological parameters, suggesting that a degree of hemolytic anemia may have been present in infected cattle (TAGAWA et al., 2010; 2012). Regarding the epidemiology of hemoplasmas infections, in general, routes of infection remain uncharacterized; although mechanical and transplacental transmissions have been suggested (HORNOK et al., 2011).

In Brazil, effects of '*Ca. M. haemobos*' occurrence and chronically infected asymptomatic carriers

on dairy production have not been investigated. The objective of this study was to describe the prevalence of '*Ca. M. haemobos*' using a PCR-based detection method and correlate this with dairy herd variables in family farms of the Ji-Paraná municipality, north region, Brazil.

MATERIALS AND METHODS

This study was conducted in the municipality of Ji-Paraná (10° 52' 42" S, 61° 56' 41" W), in the state of Rondônia. Dairy cows were sampled from September 2012 to November 2013, alongside samples taken for an alternate study on the seroprevalence of *Neospora caninum* (BOAS et al., 2015). For sample collection, the municipality area was divided into six strata of milk production, referred to as rural sectors (Figure 1), in accordance with data from the Technical Assistance and Rural Extension Association of the state of Rondônia (EMATER-RO).



Sample size was determined by the statistical formula $N_0 = [Np(1-p)/(N-1)(d/Z_{\alpha/2})^2 + p(1-p)]/deff$ with a population size of 34,527 animals, estimation of prevalence of 50%, maximum error of 6%, 95% confidence interval, and design effect (*deff*) of 1.2. The estimation of prevalence was based on no previous studies in the region and that the maximum number of occurrences with normal distribution would be 50%. The sample size was 320 animals with weighting according to the sampling design, and taking into consideration the probabilities of selection among the farms and animals (Table 1). Blood samples were collected from five >24-month-old dairy cows from each of the 64 family farms selected, and a questionnaire was given to each farmer for epidemiological data analysis.

DNA was extracted from blood samples using the phenol-chloroform method (SAMBROOK & RUSSEL, 2001) and stored at -20°C until further use. DNA samples were tested for the presence of '*Ca. M. haemobos*' using PCR analysis as previously described (GIROTTI et al., 2012). DNA from '*Ca. M. haemobos*' (GenBank accession number JN314393) and nuclease free water were used as positive and negative controls, respectively. PCR products were analyzed by electrophoresis on 1.5% agarose gels, and positive samples were purified and then sequenced in an automated sequencer (ABI PRISM® 3500).

Partial sequences were subject to Basic Local Alignment Search Tool (BLAST; ALTSCHUL et al., 1990) analysis to identify those GenBank sequences with closest similarities. A phylogenetic tree based on 460bp partial sequences of 16S rRNA was produced using the Neighbor-Joining Distance method (Figure 2). Bootstrap values, calculated from 1000 replicates, showed the percentage of replicate trees in which the associated ratio clustered together.

The phylogenetic tree, sequence alignments, and identity tables (data not shown) were created by using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (TAMURA et al., 2007).

Statistical analyses were performed using the R statistical package (R DEVELOPMENT CORE TEAM, 2013). Correlations between the dairy herds that tested positive for '*Ca. M. haemobos*' and the herd variables were analyzed by means of the chi-square (χ^2) test, with a significance of 5% applied. The considered herd variables were abortion frequency, weak calf birth rate, total cattle number, >24-month-old cow number, farm size (hectares), and production system.

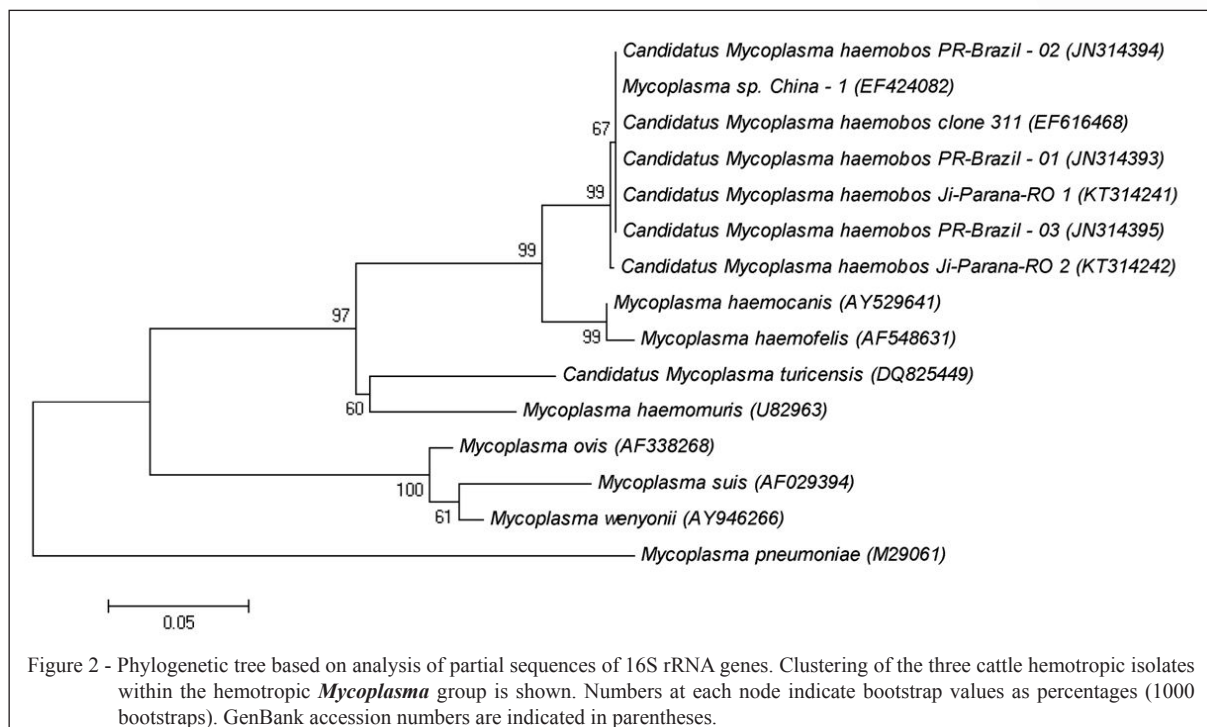
RESULTS

Overall, 207 blood samples generated 16S rRNA amplicons specific for '*Ca. M. haemobos*', which equated to 64.2% prevalence in the total population (95% CI: 58.68-69.37%). Across the six rural sectors in the municipality (Figure 1), at least one of the animals sampled from each dairy herd was positive for '*Ca. M. haemobos*', with the exception of three farms located in sectors one, four, and five. This equated to 95.3% '*Ca. M. haemobos*' prevalence in herds (61/64; Table 1).

Seven samples positive for '*Ca. M. haemobos*', drawn from each of the six rural sectors, were sequenced. Comparison of partial 16S rRNA (460bp) sequences yielded two haplotypes that differed by a single nucleotide polymorphism. BLAST analyses of these sequences showed 99-100% similarity to '*Ca. M. haemobos*' (GenBank EF616468). The 16S rRNA partial sequences generated in the present study were submitted to GenBank with the accession numbers KT314241 ('*Ca. M. haemobos*' isolate Ji-Parana/RO-1) and

Table 1 - Columns left to right: (1) Rural sector of the municipality of Ji-Paraná, Rondônia. (2) Total number of farms in the rural sector. (3) Number of farms sampled in the current survey. (4) Number of sampled farms positive for '*Candidatus Mycoplasma haemobos*' infection, and the proportion of infected farms of all sampled farms. (5) Total number of >24-month-old dairy cows in the rural sector. (6) Number of cows sampled in the current survey. (7) Number of cows positive for '*Ca. Mycoplasma haemobos*' infection, and the proportion of infected cows of all sampled cows. All data was collected from September 2012 to November 2013.

Rural sector	N. of farms	N. of farms sampled	N. of farms positive (%)	N. of dairy cows	N. of dairy cows sampled	N. of dairy cows positive (%)
1	122	9	8 (88.8)	5,246	45	26 (57.7)
2	124	9	9 (100)	5,952	45	29 (64.4)
3	54	4	4 (100)	2,268	20	12 (60.0)
4	176	13	12 (92.3)	8,096	65	36 (55.3)
5	143	11	10 (90.0)	4,433	55	35 (63.6)
6	237	18	18 (100)	8,532	90	69 (76.6)
Total	856	64	61 (95.3)	34,527	320	207 (64.2)



KT314242 (*'Ca. M. haemobos'* Ji-Parana/RO-2). Phylogenetic analysis revealed that all *'Ca. M. haemobos'* sequences clustered into a single branch (99% of bootstrap) and the sequences originating from Brazil clustered with those originating from China and Switzerland (Figure 2).

Out of the analyzed herd variables, which included abortion frequency, weak calf birth rates, total cattle number, >24-month-old cow number, farm size, and production system, only the number of >24-month-old cows correlated with *'Ca. M. haemobos'* prevalence in the surveyed farms ($P=0.03723$; Table 2).

DISCUSSION

The municipality of Ji-Paraná, Rondônia, is one of the major dairy production areas in the north region, Brazil, which makes important to evaluate infectious agents that have the potential to negatively affect productivity in the industry. In the present study, a survey using a specific PCR assay in addition to phylogenetic analysis revealed that *'Ca. M. haemobos'* infection is widespread among dairy herds in family farms, which is a particular production system in the region.

Bovine hemoplasmas are distributed worldwide, and previous studies have shown that

14.3-83.3% of cattle have chronic *'Ca. M. haemobos'* infections in Europe, Asia, and Oceania (HOELZLE et al., 2011; TAGAWA et al., 2012; MCFADDEN et al., 2016). However, epidemiological data on *'Ca. M. haemobos'* in the Americas are scarce, with only a single study in southern Brazil which reported similar infection rates as in other countries (61.0% of dairy cattle) (GIROTTI et al., 2012), so further investigation is necessary to document infection rates in cattle herds across the country.

High prevalence of *'Ca. M. haemobos'* in >2-year-old dairy cattle was expected following reports of TAGAWA et al. (2012) that 1- to 3-year-old cattle are more susceptible to bovine hemoplasmas infection. In addition, GIROTTI et al. (2012) also observed that *'Ca. M. haemobos'* detected by PCR was present at a higher rate in >2-year-old female cattle. Indeed, females stay longer in the herd and are consequently more exposed to potential transmission vectors (SMITH et al., 1990).

Furthermore, considering that the number of >24-month-old cows was the only herd variable that correlated with *'Ca. M. haemobos'* prevalence in the surveyed farms, it is notable that fewer than 50 cows existed in those farms where *'Ca. M. haemobos'* was not detected. This suggests that farms with over fifty >24-month-old females are at a higher risk of *'Ca. M. haemobos'* infection. However, this tendency was not observed in farms with

Table 2 - Correlation between '*Candidatus Mycoplasma haemobos*' prevalence in dairy cows detected by PCR and the analyzed herd variables.

Variables analyzed	-----Presence of ‘ <i>Ca. M. haemobos</i> ’-----		P-value
	Positive	Negative	
-----Number of cows-----			
<50	7	2	0.0605
51 to 100	19	1	
101 to 150	15	0	
>150	20	0	
-----Number of cows aged over 24 months-----			
<25	7	2	0.0372*
25 to 50	18	1	
51 to 100	31	0	
>100	5	0	
-----Creation system-----			
Extensive	50	2	0.4696
Semi-Extensive	9	1	
Semi-Intensive	2	0	
-----Size of farm (hectares)-----			
<25	10	1	0.8609
25 to 50	20	1	
51 to 100	23	1	
>100	8	0	
-----Occurrence of abortion-----			
Yes	35	1	0.5766
No	26	2	
Presence of weak calves			
Yes	26	0	0.3046
No	35	3	

*Presence of statistical significance.

more than 100 cows, possibly due to the low proportion (5/64) of analyzed farms in this category.

A greater proportion of >24-month-old animals can increase the likelihood of infected animals in the herd and, as a consequence of a high presence of infection, transmission of '*Ca. M. haemobos*' via vectors to susceptible dairy cows (SMITH et al., 1990). However, no data currently exists on the involvement of potential vectors in the transmission of '*Ca. M. haemobos*' in Brazil, such as dipterans (e.g., *Stomoxys calcitrans* and *Haematobia irritans*) as described by HORNOK et al. (2011), and the cattle tick (*Rhipicephalus microplus*). Thus, the involvement of transmission vectors in '*Ca. M. haemobos*' herd infection rates requires further investigation.

CONCLUSION

A PCR-based detection method showed that '*Ca. M. haemobos*' prevalence is widespread among dairy herds in the municipality of Ji-Paraná,

Rondônia, mainly in farms with over fifty >24-month-old females, which expands distribution of the bacterium in the Americas to the north region, Brazil. Considering the importance of the dairy industry to the areas surveyed in this study, further investigation is necessary to evaluate the effect of these chronically infected animals on milk production and herd health.

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BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

Bioethical Committee for Animal Research of the Universidade Federal do Mato Grosso approved the present study, under protocol n. 23108.015662/12-5.

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