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Molecular detection and occurrence of '*Candidatus* Mycoplasma haemobos' in dairy cattle of Southern Brazil

Detecção molecular e ocorrência de '*Candidatus* Mycoplasma haemobos' em bovinos de leite do Sul do Brasil

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Abstract

Bovine hemoplasmas are bacteria found on the erythrocyte surface or free in the plasma of cattle. The aim of the present study was to evaluate the occurrence of '*Candidatus* Mycoplasma haemobos' ('*C.* M. haemobos') in Holstein and Jersey cattle raised in Londrina and surroundings, northern region of the State of Parana, Southern Brazil. PCR testing directed to 16S rRNA gene fragment was performed to investigate the occurrence and characterize the molecular identity of '*C.* M. haemobos'. A total of 264/433 (60.97%) blood samples were positive by PCR. Further alignment of 500-bp amplicons to available sequences at the GenBank database showed high identity (100%) to '*C.* M. haemobos'. To the author's knowledge, this is the first molecular confirmation of the hemoplasma '*C.* M. haemobos' in cattle from Brazil. Moreover, '*C.* M. haemobos' was observed in high occurrence in dairy cattle, and may have significant impact in livestock production.

Keywords: *'Candidatus* Mycoplasma haemobos', bovine, occurrence, phylogenetic tree, Brazil.

Resumo

Hemoplasmas de bovinos são bactérias encontradas na superfície de hemácias, ou livre no plasma de bovinos. O objetivo do presente estudo foi avaliar a ocorrência de *Candidatus* Mycoplasma haemobos' ('*C.* M. haemobos') em bovinos das raças Holandesa e Jersey da região de Londrina, norte do Paraná, sul do Brasil. Para investigar a ocorrência e caracterizar a identidade molecular do '*C.* M haemobos' uma PCR baseada no fragmento do gene 16S rRNA foi realizada. A PCR identificou como positivas 264/433 (61%) amostras de sangue testadas. O alinhamento deste fragmento de 500 pb com seqüências disponíveis no GenBank mostrou 100% de identidade '*C.* M. haemobos'. Pela bibliografia consultada, esta é a primeira confirmação molecular do hemoplasma '*C.* M. haemobos' em bovinos no Brasil. Além disso, foi observada uma alta prevalência deste hemoplasma em bovinos de leite, que pode ter um impacto importante na pecuária bovina.

Palavras-chave: '*Candidatus* Mycoplasma haemobos', bovinos, árvore filogenética, ocorrência, Brasil.

Hemotropic mycoplasmas, also known as hemoplasmas, are cell wall-less organisms that attach to erythrocytes of a variety of domestic and wild animal species including human beings (MESSICK, 2004; SANTOS et al., 2008). In cattle, two distinct hemotropic *Mycoplasma* have been identified to date: *Mycoplasma wenyonii* (formerly *Eperythrozoon wenyonii*) (ADLER; ELLENBOGEN, 1934; SUTTON, et al., 1977) and '*Candidatus* Mycoplasma haemobos' ('*C.* M. haemobos') (TAGAWA et al., 2008).

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'*Candidatus* Mycoplasma haemobos' has been reported using molecular methods such as polymerase chain reaction (PCR) and sequencing techniques in cattle from Switzerland, Germany, China, and Japan (HOFMANN-LEHMANN et al., 2004; TAGAWA et al., 2008; SU et al., 2010; HOELZLE et al., 2011). However, to the author's knowledge, no molecular detection has been reported to date in the Americas. Accordingly, the aim of the present study was to evaluate the occurrence of '*C.* M. haemobos' in Holstein and Jersey cattle raised in Londrina and surroundings, northern region of the State of Parana, Southern Brazil.

A total of 433 blood samples from dairy cattle (Holstein and Jersey) were collected between July 2009 and June 2010. Information relative to the sex, age and hematocrit of all animals was also obtained

and included in this study. Samples were drawn by jugular venopuncture, immediately placed in EDTA tubes and stored at –20 °C prior to DNA extraction. Total Genomic DNA was extracted from 200 µL of each blood sample with a commercially available kit (DNeasy Blood & Tissue Kit, QIAGEN, Hilden, Germany) and stored at –20 °C until PCR testing. PCR was carried out using the primers (5'-ATC TAA CAT GCC CCT CTG TA-3'/5'-GTA GTA TTC GGT GCA AAC AA-3') as previously described (NISHIZAWA et al., 2010) with a few modifications: one microliter of DNA and 10 pmol of each primer were used in a 12.5 µL PCR total volume, and an increase of 6 °C for the annealing temperature was applied to improve the test specificity. DNA from '*C.* M. haemobos' and nuclease free water were used as positive and negative controls, respectively. The sensitivity of the PCR was evaluated by using a serial 10-fold dilution of DNA in water showing specific visible band until 10^{-6} dilution. The detection limit was 10 fg of genomic DNA. To evaluate the specificity of the '*C.* M. haemobos' - PCR, DNA extracted from *Mycoplasma haemocanis*, *Mycoplasma haemofelis*, *Anaplasma marginale*, *Anaplasma centrale* were used as DNA templates. PCR products were analyzed on 1.5% Agarose gel stained with commercial gel stain (SYBR® Safe DNA, Invitrogen, Eugene, OR, USA). The 100 bp ladder (Invitrogen, Eugene, OR, USA) was used as standard to initially determine the molecular mass of PCR products. The presence of DNA integrity and absence of PCR inhibitors in extracted samples that tested negative in PCR were confirmed by the successful amplification of a housekeeping gene fragment (glyceraldehyde-3-phosphate dehydrogenase - GAPDH) (BIRKENHEUER et al., 2003). Amplicons with the expected size were purified (QIAquick Gel Extraction Kit, QIAGEN, Hilden, Germany) and submitted to direct sequencing using a commercial sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA); sequences were submitted to BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda, MD, 20894, USA) to determine identity to other hemotropic *Mycoplasma* species. Epi info™ software was used to evaluate differences between variables; $p \leq 0.05$ was considered as significant.

In the present study, DNA fragment of approximately 500 bp of the 16S rRNA gene of '*C.* M. haemobos' was amplified from 264/433 blood samples of dairy cattle. There was no amplification of DNA of any other agents used as specificity controls for '*C.* M. haemobos'. Direct sequencing of PCR amplicons from three representative samples confirmed that the amplified partial 16S rRNA (368bp) sequence represented '*C.* M. haemobos', ranged from 98 to 100% of identity with the known sequences in the GenBank database (EF616468, EF424082, EU367965, EF460765, EF616467), confirming the specificity of the PCR. The sequences were deposited in the GenBank database under accession numbers JN314393, JN314394 and JN314395. Moreover, the present results have shown a high occurrence of '*C.* M. haemobos' in dairy cattle from Southern Brazil, with 61.0% of positive animals.

A phylogenetic tree based on partial sequences of 16S rRNA genes (368 bp) found was produced applying the Neighbor-Joining method (Figure 1). The bootstrap values, calculated from 1000 replicates, show 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 4.0 (TAMURA et al., 2007).

Categorical variables were compared between PCR-positive and negative cattle using chi-square test. The hemoplasma-infected cattle in the present study did not appear to be clinically affected; no statistically significant differences in hematocrit were found between PCR-positive (ranged from 20% to 47%, average = 31.76 ± 5.21) and PCR-negative (ranged from 19% to 43%, average = 31.52 ± 4.95) animals ($p = 0.054$). Furthermore, no statistical differences were observed for anemic animals ($p = 0.445$). Animals above two years old ($p = 0.001$), and females ($p = 0.002$) presented higher occurrence of positive PCR. These results are consistent with the fact that females stay longer in the

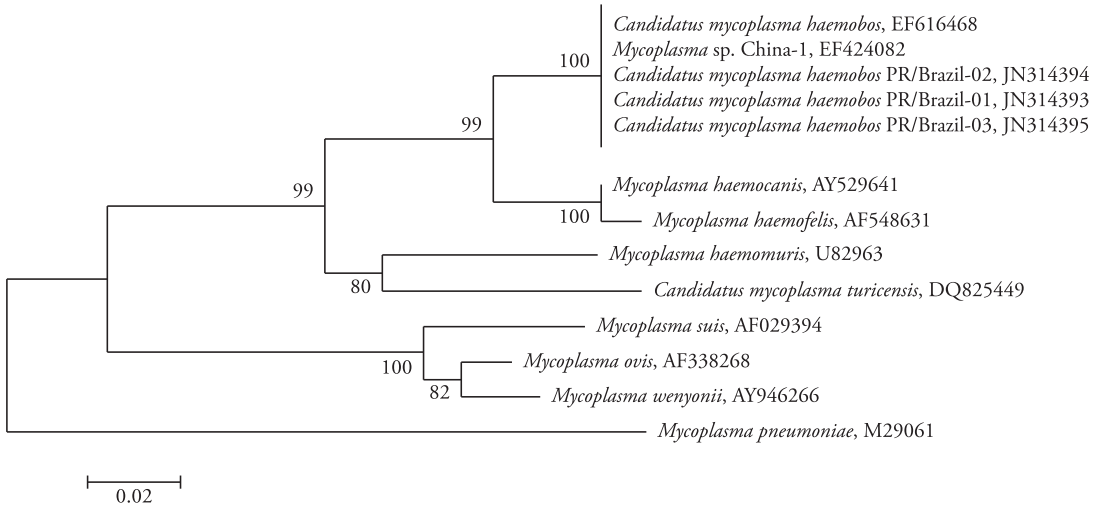

Figure 1. Phylogenetic tree based on partial sequence analysis of 16S rRNA genes showing the clustering of the three cattle hemotropic isolates among the hemotropic *Mycoplasma* group. The tree shown was generated applying the Neighbor-Joining method (MEGA 4.1 software; TAMURA et al., 2007). *Mycoplasma pneumoniae* was used as out-group. The numbers at the nodes indicate bootstrap values in percent (1000 bootstraps). Numbers in brackets are GenBank accession numbers.

herds and, consequently, are more exposed to potential vectors (SMITH et al., 1990). Considering the frequency of vectors from our region in cattle, such as, *Rhipicephalus* (*Boophilus*) *microplus* and *Stomoxys calcitrans*, these species could be involved in the transmission of this pathogen; however, this observation needs further investigation.

The 61.0% of positive animals reported in this study represents the first epidemiological information of cattle infected with '*C*. M. haemobos' in Brazil. The epidemiological studies of cattle infected with this hemoplasma are from Hungary (HORNOK et al., 2012) and Switzerland (MELI et al., 2010), where 87.5% of animals were found positive during an outbreak of bovine anaplasmosis and 65.4% of fatal cases were of anemic cows, respectively.

It is important to emphasize that in the present study, the animals were apparently healthy, and there was no significant statistical difference between hematocrit and positive and negative animals. Meli et al. (2010) found that the majority of positive animals were healthy; this result was similar to the findings of this research, where 50.8% of healthy animals were also positive for '*C*. M. haemobos'. In the present study, it was not possible to determine whether the positive animals for '*C*. M. haemobos' had presented the disease in the past, especially because the region studied presents a high incidence of anaplasmosis and other hemoparasites in the herds (ANDRADE et al., 2001).

Although the results indicate high occurrence of '*C*. M. haemobos' within the healthy dairy cattle population of Northern Parana, Southern Brazil, infected animals may represent chronically asymptomatic carriers and the impact on dairy production is yet to be established. In addition, the results might have important implications on further studies regarding the pathogenicity and molecular epidemiology of '*C*. M. haemobos'. To the author's knowledge, this is the first report of detection and occurrence of '*C*. M. haemobos' in dairy cattle in Brazil based on molecular evidence.

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