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Detection of clinical bovine mastitis caused by *Mycoplasma bovis* in Brazil

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Research Article

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Abstract

The work reported in this research communication investigated the occurrence of *Mycoplasma bovis* (*M. bovis*) in milk samples from cows with clinical mastitis on dairy farms from seven Brazilian states. We hypothesized that *M. bovis* was present in bovine clinical mastitis milk in Brazil. A total of 561 milk samples were cultured on Hayflick agar and incubated in a microaerophilic atmosphere at 5% CO_2. Polymerase chain reaction (PCR) was performed for the detection of *Mycoplasma* spp. and *Mycoplasma bovis* in milk samples. *Mycoplasma* spp. were isolated in 2% of the milk samples, and *Mycoplasma bovis* was verified in 3% of the milk samples by PCR. The results showed that *Mycoplasma bovis* is involved in clinical mastitis in Brazilian dairy herds. We emphasize the need for further studies to investigate the infection by this agent in clinical mastitis cases, particularly in Brazil, due to the lack of knowledge about its prevalence.

Microorganisms of the genus *Mycoplasma* have a worldwide distribution and can cause different diseases in infected animals. The course of mycoplasma infection is determined by the virulence and dose of the strain and time of exposure to the pathogen. Mycoplasma infection can cause pneumonia, polyarthritis and mastitis (Pfützner and Sachse, 1996).

Mastitis caused by *Mycoplasma* spp. is relatively common in large dairy herds (Pfützner and Sachse, 1996). The prevalence of this pathogen, however, is probably underestimated (Nicholas and Ayling, 2003), particularly in Brazil, where there are few reports of its involvement in mastitis. This may be because there are few laboratories that perform routine *Mycoplasma* spp. analysis. The isolation of this agent requires special conditions and selective culture media.

Although several *Mycoplasma* spp. have been isolated as causative agents of bovine mastitis, *Mycoplasma bovis* is the most important species due to its high pathogenicity and prevalence in dairy herds (González and Wilson, 2003). *M. bovis* may cause clinical, subclinical or chronic mastitis. Once mastitis is established, the affected animal can release 10^6 to 10^8 CFU of *M. bovis* per ml of milk, contaminating the milking machine, milker's hands and latex gloves and other utensils. Milk is, therefore, a significant transmission vector to other animals in the herd (González and Wilson, 2003; Houlihan *et al.*, 2007).

To control *M. bovis* mastitis, it is necessary to test the entire herd to detect infected animals. Once the infected animals are detected, they must be slaughtered or isolated. Simultaneously, preventive measures should be established for contagious mastitis, such as milking hygiene, immersion of teats in antiseptic solution after milking, and disinfection and maintenance of milking equipment (Houlihan *et al.*, 2007). Considering the probable underdiagnosis and the unknown economic and health impacts of *M. bovis* as a causative agent of bovine mastitis in Brazil, the present study was undertaken to verify the occurrence of *Mycoplasma* spp. and *M. bovis* in milk samples from cows with clinical mastitis in Brazil.

Materials and methods

This research was approved by the Ethics Committee on Animal Use of the São Paulo State University (Unesp) CEUA 94/2016.

Animals

Five hundred and sixty-one ($n = 561$) milk samples from individual quarters of cows with clinical mastitis were used in this study, and the diagnosis of clinical mastitis was based on macroscopic changes in the milk appearance including the presence of lumps, pus or blood streaks.

Milk samples were collected in sterile tubes after teat antisepsis with 70.0% alcohol. Milk samples were collected from the following Brazilian states: Ceará 1.4% (8/561), Goiás 6.4% (36/561), Minas Gerais 39.0% (219/561), Paraná 12.1% (68/561), Rio Grande do Sul 2.0% (11/561), Rio de Janeiro 1.1% (6/561) and São Paulo 38.0% (213/561). The evaluated milk samples were sent to the Mastitis Research Center (NUPEMAS), São Paulo State University (Unesp), in Botucatu, São Paulo, for routine microbiological diagnosis of the dairy herds.

Dairy herds were classified according to the number of lactating cows as follows: small = less than 100; medium = 101 to 350; large = more than 351. Due to the sampling method used, it was not possible to obtain herd size data for two properties, which were classified as 'unknown'.

Microbiological culture for Mycoplasma *spp.*

After slow thawing, all samples were tested by culture and by PCR. Milk samples were cultured on plates containing Hayflick with 0.01% thallium acetate and incubated in a microaerophilic atmosphere at 5% CO_2 for 15 d. Microscopic visualization of the colonies was performed every three days, from the third to the fifteenth day of culture, and colonies that presented the characteristic feature of a 'fried-egg' were selected (Pretto *et al.*, 2001).

DNA extraction

DNA extraction from milk samples was performed with an Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's specification, with modifications: the milk sample (200 µl) was centrifuged at 1000 ×*g* to remove milk fat, and this centrifugation step was repeated to remove the supernatant.

PCR amplification

To amplify a 270 bp DNA fragment from the Mollicutes class, PCR was performed with the primers *MGSO* (5′ TGC ACC ATC TGT CAC TCT GTT AAC CT 3′) and *GPO-3* (5′ GGG AGC AAA CAG GAT TAG ATA CCC T 3′).

Positive samples for *Mycoplasma* spp. were subjected to PCR with specific primers for *M. bovis*, *MBOr* (5′ CCG TCA AGG TAG CAT CAT TTC CTA T 3′) and *MBOf* (5′ CCT TTT AGA TTG GGA TAG CGG ATG 3′).

PCR was performed in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany), and the amplified products were visualized by 2% agarose gel electrophoresis stained with Nancy (Merck KGaA, Darmstadt, Germany). The electrophoresis image was recorded and analysed.

Results and discussion

From 561 milk samples cultured in specific medium for *Mycoplasma* spp., 2.0% (11/561) had colonies suggestive of *Mycoplasma* spp. Based on microscopic visualization, 81.8% (9/11) of the colonies obtained on the third day were positive. The remaining two samples were positive on the sixth day of observation.

Molecular detection of *Mycoplasma* spp. in the Mollicutes class was observed in 3.0% (17/561) of the milk samples. These samples were also positive by PCR with specific primers for *Mycoplasma bovis*. Table 1 describes the distribution of positive samples according to the herd size and state of origin of the cows.

Intramammary mycoplasmosis in Brazil has a prevalence rate that is most likely underestimated due to the lack of data about this infection. Its real damage and impact to the dairy herds of Brazil was, therefore, unknown. The present study was developed to provide the scientific community with more comprehensive data on the prevalence of clinical mastitis caused by *Mycoplasma*, particularly considering that research on the aetiological agents involved in clinical mastitis has generally not considered *Mycoplasma*. We observed that 3.0% of clinical mastitis in cows was caused by *M. bovis* in four Brazilian states. This causative agent was described by González and Wilson (2003). *M. bovis* was described as the most frequent species of the genus *Mycoplasma* spp. causing mastitis in dairy herds, which was corroborated with the results obtained in the present study; 17 milk samples from the Mollicutes class that were positive for *Mycoplasma* spp. based on PCR were also positive with specific *M. bovis* primers. Thus, the results reaffirm the pathogenicity of *M. bovis* in the aetiology of clinical mastitis.

Table 1. Distribution of milk samples obtained from cows with clinical mastitis and positive for *Mycoplasma bovis*, as determined by molecular amplification, according to the state, number and size of herd of origin

State (Brazil)	Herds	Herd size	Number of positive samples *N* (%)
Paraná	A	Large	6/17 (35.3%)
Goiás	B	Medium	3/17 (17.6%)
São Paulo	C	Unknown	1/17 (5.9%)
	D	Large	4/17 (23.5%)
Minas Gerais	E	Unknown	2/17 (11.8%)
	F	Medium	1/17 (5.9%)
Ceará	G	Medium	0
Rio de Janeiro	H	Medium	0
Rio Grande do Sul	I	Large	0

In the present study, 35.0% ($n = 6$) of the samples that were positive in microbiological culture in Hayflick medium were negative by PCR. Justice-Allen *et al.* (2011) compared these two methods in the detection of *M. bovis* in 180 milk samples from bulk tanks and verified a concordance of 80%. No positive sample was considered a false positive, therefore, in both tests, the specificity was considered 100%, while the sensitivity was 76.7 and 68.9% for PCR and culture, respectively.

Due to the sampling method adopted in this research, the production system of the properties involved and the fact that the size of two positive herds for *M. bovis* were unknown, it was difficult to analyse the possible risk factors that could trigger the infection in detail and create hypotheses. However, the main concern of this research was to provide information about the occurrence and prevalence of clinical mastitis caused by *M. bovis* in herds from different Brazilian states, emphasizing that, to our knowledge, only one work was developed with this objective in Brazil, which was conducted in milk herds from Paraná state (Pretto *et al.*, 2001). The low prevalence in our study agrees with theirs, since they reported that 1.1% (8/713) of clinical mastitis in cows from three dairy farms in Paraná state, southern Brazil, was associated with *M. bovis* infection. The authors also pointed to the need to implement *Mycoplasma* diagnosis as a routine laboratory practice.

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The occurrence of *M. bovis* as a pathogenic aetiology of mastitis is variable in several countries, with 0.5 to 55% of the cases reported (González and Wilson, 2003; Miranda-Morales *et al.*, 2008). The variability in the occurrence of *M. bovis* in different countries may be related to the techniques used for the diagnosis, size of the herds and routine culture of the agent in diagnostic laboratories (González and Wilson, 2003). Reports of the isolation of *Mycoplasma* spp. in the US are variable. In the state of Utah, the presence of *Mycoplasma* spp. was reported in 7% of the herds examined, whereas in Washington, 20% of the herds were positive for *Mycoplasma* spp. (Wilson *et al.*, 2009). The average size of US herds is 187 animals (USDA, 2017), which probably influences the higher prevalence of this pathogen in this country. It is known that the contagiousness of mycoplasmas is high and there is a possibility of contamination during the milking process (González and Wilson, 2003). In this study, among the positive samples from herds of known size, only cows from medium or large herds were positive for *Mycoplasma* spp. It should be emphasized that animal health management is extremely important for the control of mastitis, including cases of mastitis by mycoplasmas.

The correct identification of the causative agent involved in clinical mastitis allows the adoption of strategic control measures. The identification of a specific infectious agent in clinical mastitis cases indicates that it is also likely involved in subclinical mastitis. Thus, it is possible to investigate the detected agent in individual quarter milk to determine if the milk should be discarded. It is especially important in mastitis caused by *Mycoplasma* since the prospects of treatment success are low (Houlihan *et al.*, 2007).

In conclusion, this work demonstrated the involvement of *Mycoplasma bovis* in clinical mastitis cases in cows from dairy herds from four Brazilian states, with a prevalence of 3.0%, as determined by molecular detection. Since the methods employed in this study were useful for identifying *M. bovis* infection, they can be used in further studies to elucidate the prevalence of bovine clinical mastitis caused by this agent and its economic and health impact, particularly in Brazil, where this information is still scarce.

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