PRESENÇA DE *Mollicutes* E *Mycoplasma bovis* EM SWABS NASAIS DE BEZERROS E NA SECREÇÃO MASTÍTICA DE VACAS

Brunna Mayla Vasconcelos Adorno[1]
Anelise Salina[1]
Sâmea Fernandes Joaquim[1]
Felipe de Freitas Guimarães[1]
Bruna Churocof Lopes[1]
Benedito Donizeti Menozzi[2]
Helio Langoni[3]

RESUMO

Mycoplasma bovis faz parte da microbiota do trato respiratório bovino, mas é considerado um patógeno oportunista de extrema importância nas doenças respiratórias de bezerros. Causa ao rebanho diversas doenças como mastite, poliartrite, pneumonia e endometrite. Esse patógeno é altamente contagioso e os animais com mastite são potenciais disseminadores da infecção para o rebanho, pois liberam de 10^6 a 10^8 UFC por mL de leite. Da mesma forma, animais com pneumonia eliminam, por meio de secreções respiratórias, altas cargas microbianas do agente. O presente estudo teve como objetivo realizar a detecção molecular de *Mycoplasma bovis* em 185 amostras de secreção mastítica de vacas, bem como em 50 amostras de swab nasal de bezerros saudáveis com ou sem sinais de pneumonia e nascidos de vacas com mastite, pertencentes a quatro fazendas leiteiras do estado do Paraná, onde foram diagnosticados casos de mastite. A extração do DNA de ambas as secreções foi realizada pelo método de termólise. Para a reação em cadeia da polimerase (PCR), primers genéricos foram empregados para amplificar o DNA dos *Mollicutes* e as amostras positivas foram submetidas à PCR com primers específicos para *M. bovis*. A positividade para *M. bovis* foi de 3,78% nas amostras de secreção mastítica, independente da fazenda, e de 20% nos swabs nasais.

Palavras-chave: mastite, doenças respiratórias, PCR

PRESENCE OF *MOLLICUTES* AND *MYCOPLASMA BOVIS* IN NASAL SWABS FROM CALVES AND IN MASTITIC SECRETION FROM COWS

ABSTRACT

Mycoplasma bovis is part of the bovine respiratory tract microbiota but is considered an opportunistic pathogen of extreme importance in respiratory diseases of calves. It causes to the herd several diseases such as mastitis, polyarthritis, pneumonia and endometritis. This pathogen is highly contagious and animals with mastitis are potential disseminators of infection to the herd since they release from 10^6 to 10^8 CFU per mL milk. Similarly, animals with pneumonia eliminate, through respiratory secretions, high microbial loads of the agent. The present study aimed to perform molecular detection of *Mycoplasma bovis* in 185 milk samples from cows with clinical mastitis, as well as in 50 nasal swab samples from healthy calves with or without signs of pneumonia and born from cows with mastitis, all belonging to four dairy farms in Paraná State, where cases of mastitis had been diagnosed. DNA extraction

[1] Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, brunnamaylav@gmail.com
[2] Assistente de Suporte Acadêmico III, Departamento de Higiene e Veterinária e Saúde Pública, FMVZ-UNESP Botucatu benedito.menozzi@unesp.br
[3] Professor Titular do Departamento de Higiene e Veterinária e Saúde Pública, FMVZ-UNESP Botucatu. Correspondencia: helio.langoni@unesp.br

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from both secretions was carried out according to the thermolysis method. For polymerase chain reaction (PCR), generic primers were employed to amplify the *Mollicutes* DNA and positive samples were subjected to PCR with primers specific for *M. bovis*. Positivity for *M. bovis* was 3.78% in mastitic samples, regardless of the farm, and 20% in nasal swabs.

Keywords: mastitis, respiratory diseases, PCR

PRESENCIA DE *MOLLICUTES* E *MYCOPLASMA BOVIS* EN HISOPOS NASALESES DE TERNEROS Y SECRECION DE MASTITIS DE VACAS

RESUMEN

Mycoplasma bovis forma parte de la microbiota del tracto respiratorio bovino, pero se considera un patógeno oportunista de extrema importancia en las enfermedades respiratorias de los terneros. Provoca en el rebaño diversas enfermedades como mastitis, poliartritis, neumonía y endometritis. Este patógeno es altamente contagioso y los animales con mastitis son potenciales diseminadores de la infección al hato, ya que liberan de 10^6 a 10^8 UFC por ml de leche. Asimismo, los animales con neumonía eliminan, através de secreciones respiratorias, altas cargas microbianas del agente. El presente estudio tuvo como objetivo realizar la detección molecular de *Mycoplasma bovis* en 185 muestras de secretion de mastitis de vacas con mastitis clínica, así como en 50 muestras de hisopos nasales de terneros sanos con o sin signos de neumonía y nacidos de vacas con mastitis, todos pertenecientes a cuatro granjas lecheras en el estado de Paraná, donde se diagnosticaron casos de mastitis. La extracción de ADN de ambas secreciones se realizó mediante el método de termólisis. Para la reacción en cadena de la polimerasa (PCR), se utilizaron cebadores genéricos para amplificar el ADN de los *Mollicutes* y las muestras positivas se sometieron a PCR con cebadores específicos para *M. bovis*. La positividad para *M. bovis* fue del 3,78% en muestras de mastitis, independientemente de la granja, y del 20% en hisopos nasales.

Palabras clave: mastitis, enfermedades respiratorias, PCR

INTRODUCTION

The bovine respiratory complex is multifactorial and has multiple etiology, constituting one of the major problems in cattle raising, especially for calves. It is responsible for high morbidity and lethality rates and leads to great economic losses due to high treatment costs, reduced yield and animal weight gain (1).

Occurrence of pneumonia in calves has as determining factors the infectious agents and as predisposing factors the stress, the environmental conditions and the host's immunity. A large number of microorganisms can cause respiratory problems to these animals, including bacteria, viruses, fungi and protozoa, of which bacteria and viruses are most frequent; among bacterial agents, *Mycoplasma spp*. are most common, especially *Mycoplasma bovis* (1,2).

Other species are considered relevant for the etiology of bovine pneumonias, such as *Mycoplasma mycoides* subspecies mycoide Small Colony (SC) and *Mycoplasma dispar*. The first causes contagious bovine pleuropneumonia, while *M. dispar* has been isolated from the lungs of diseased calves; however, it can also be isolated from healthy animals, which constitute sources of infection to the herd (3,4).

Mycoplasma bovis is part of the bovine respiratory tract microbiota and is present in the mucosae of the urogenital and gastrointestinal tracts, eyes and mammary glands. This primary

and opportunistic pathogen is considered the second most important species in respiratory diseases of calves and the most frequent pathogen in bronchopneumonia of calves (4-6).

Recently acquired female calves or heifers, asymptomatic but carriers of *M. bovis*, are the main sources of infection to free herds. Once introduced into the herd, eradication is extremely difficult. Thus, quarantine and clinical and microbiological evaluation of animals before incorporation into the herd must be part of control programs (7,8), and also the disposable of infected animals.

Some species present tropism for specific regions and others for diverse sites, such as *M. bovis*, which may cause different conditions like: mastitis, severe pneumonia in calves, polyarthritis, fibrous suppurative and necrotizing tenosynovitis, keratoconjunctivitis, otitis media, endometritis, salpingitis, abortion and seminal vesiculitis (9). Transmission to other animals can be directly by the contact with contaminated respiratory secretion, like in the nostril-nostril contact, and indirectly by the ingestion of contaminated water and food, inhalation of aerosol particles and contaminated fomites (5).

An important transmission route for calves is milk contaminated with *M. bovis*, relevant in the disease epidemiology. According to Bennett and Jasper (10), calves fed on contaminated milk showed higher nasal colonization rates. Bacteria of the genus *Mycoplasma* have been reported to participate in bovine respiratory problems and have been found in healthy animals. Arcangioli et al. (11) analyzed, in France, 135 calves from nine feedlots during natural occurrence of respiratory diseases and could isolate *M. bovis* from eight (89%) out of nine herds and from 106 (78.5%) out of 135 bronchoalveolar wash samples. In Netherlands, Terlaak et al. (4) found 36 calves positive for *M. bovis* when investigating the agent in tracheal washes from seven beef cattle herds with respiratory problems. Nicholas (12) reported *M. bovis* isolation from 13 to 23% cases of pneumonia in Ireland. Evaluating the etiology of pneumonias in cattle in Scotland, Hotchkins et al. (13) found 9.4% samples positive for mycoplasma in 36.7% assessed herds.

In 18 Finnish herds, of which 10 were breastfeeding units (48 to 217 animals) and eight were dairy units (30 to 130 animals), tracheal washes from 84 calves were investigated for the presence of pathogens causing respiratory problems. *Mycoplasma dispar* was detected in 40.5% samples, while other mycoplasmas species were found in 60.7% samples (14).

Dispersal of *Mycoplasma bovis* is documented. Of milk samples from tanks in 871 farms, 7.9% were positive for mycoplasma, and 86% cases were positive for *M. bovis*. Fulton et al. (15) reported 80% prevalence in the analyzed dairy farms and in 199 animals, isolating *M. bovirhinus* and *M. bovis* at a greater frequency and *M. arginini* less frequently.

Prevalence of *Mollicutes* species and *M. bovis* in nasal swabs from recently transported calves was evaluated by White et al. (16) using PCR. At their arrival to the farm, 22 (7.6%) out of 291 animals had nasal swab cultures positive for *Mollicutes* species. Animals that showed respiratory problems after their arrival had secretion collected, and from 130 calves with pneumonia, 34 (26.2%) were positive for *Mollicutes*, of which 7 (20.6%) were *M. bovis* according to PCR, reinforcing its importance in the etiology of pneumonia in calves.

In Egypt, Ghoneim et al. (17) recovered *Mycoplasma spp*. from 46.67% calves with pneumonia and 32% healthy calves. *M. bovis* was most frequent, followed by *M. dispar*.

Since it is highly contagious, *Mycoplasma bovis* disseminates among the animals. It is an important pathogen in mastitis and, besides respiratory problems, arthritis may occur in the animals, especially in calves. Cows with mastitis due to this agent are potential disseminators of the infection to other animals. In Brazil, mastitis by *M. bovis* was first reported in the region of Londrina, Paraná State, by Mettifogo et al. (18) and subsequently in farms located in the western region of São Paulo State, where calves were also diagnosed with pneumonia and arthritis (19).

Mycoplasma bovis and *Mycoplasma californicum* are most frequently involved in mastitis. *M. bovis* affects especially bovine animals, but cases in bulls and small ruminants were already reported. An animal can release a large quantity of the agent, around 10^6 to 10^8 CFU per mL milk, constituting an important source of contamination of fomites, such as milking equipment, milker's hands, intramammary cannulas and syringes (20,21). Although different species of mycoplasmas have been isolated from cattle, *M. bovis* is prevalent, reaching around 70% or more in countries in Europe, North America and Oceania (22).

Diagnosis of *M. bovis* based on microbiological culture demands specific microaerophilic conditions, enriched media, special incubation period, as well as availability of specialized routine labs. Thus, research about this agent for both mastitis and other diseases is less frequent. Even under ideal conditions, the type of clinical sample, the collection and transportation methods, the nutritional demand and the viable microbial load in the clinical material interfere in the result (23).

There is the need for alternative methods to detect this pathogen. Molecular techniques, such as polymerase chain reaction (PCR), have good accuracy for revealing specific and unique aspects of the genome of each mycoplasma species. Several studies have demonstrated the efficacy and the reliability of this method to detect *Mycoplasma spp*. in nasal secretion and in milk samples, including treated milk (23,25).

Considering that mycoplasmas are important for the etiology of respiratory problems in calves and can be part of the respiratory tract microbiota of these animals, the present study aimed to: i) detect, by using the PCR technique, the presence of *Mollicutes* spp. and *Mycoplasma bovis* in nasal swabs from both calves that were healthy or had pneumonia and in mastitis secretion from cows with clinical mastitis. ii) confirm the participation of this pathogen in bovine mastitis and that it is part of the pulmonary microbiota of calves.

MATERIAL AND METHODS

Analysis included 185 mastitic secretion from cows with clinical mastitis and 50 nasal swabs from calves. In both cases, convenience samples were used from four farms in Paraná State, named A, B, C and D, accounting for 76, 73, 28 and 8 milk samples and 22, 9, 10 and 9 nasal swabs, respectively. Included were animals with one or more signs of clinical mastitis – from light to moderate and even severe mastitis with, edema, redness, high temperature of the mammary gland, grumes and/or pus in the milk.

The teat was washed with water and the teat ostium was disinfected with iodine-alcohol solution at 0.25%, while 10-mL mastitic secretion were collected into sterile flasks. The samples were frozen at -20 centigrade degrees until 15 days and transported to the laboratory under refrigeration temperature, in boxes of isothermal material containing recyclable ice.

Nasal secretion was collected at the same time that was obtained the mastitic secretion, with sterile cotton swabs by deep introduction and friction into the nasal mucosa; then, the material was stored in tubes containing 2ml culture medium (MEM containing 5x the normal concentration of penicillin and streptomycin), homogenized and preserved as the mastitic samples and sent together to the laboratory, also under refrigeration temperature.

DNA of the samples, was extracted according to the thermolysis method (26). DNAse- and RNAse-free microtubes received 1-mL aliquot of each mastitic secretion, as well as of the transport medium containing the nasal swab, which was rubbed off the tube wall. Then, centrifugation was performed in a microcentrifuge at 10000*g* for 10 minutes, the supernatant was discarded and 500µl buffered saline solution (150 mM PBS pH 7.2) were added and centrifuged at 10000*g* for 10 minutes. After discarding the supernatant, 50 µl elution buffer were added.

Samples were kept in water bath at 100ºC for 10 minutes, immersed in ice for 5 minutes and subsequently centrifuged at 10000*g* for 10 minutes. The supernatant was stored in DNAse- and RNAse-free microtubes at 4ºC during 24 hours for DNA stabilization and frozen at -20ºC until amplification.

Molecular detection of *Mollicutes* and *Mycoplasma bovis* employed primers that amplify species-specific DNA regions that codify the regions 16S and 23S rRNA based on the sequences at GenBank. PCR reactions were carried out with a total volume of 25µL containing reaction buffer 10mM Tris-HCl pH 8.0, 50mM KCl, 1.5mM MgCl2, 0.2mM dNTP, 10ρM each primer, 0.5 units Taq Platinium (Invitrogen) and 10ng DNA, in a Mastercycler gradient thermocycler (Eppendorf).

For *Mollicutes* DNA amplification, the employed primers were MGSO (5´ TGC ACC ATC TGT CAC TCT GTT AAC CTC 3´) and GPO-3 (5´ GGG AGC AAA CAG GAT TAG ATA CCCT 3´), with common 270bp product and cycle profile as follows: five minutes at 94°C, thirty-five cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes (27).

If the PCR with generic primers resulted positive, the DNA of *Mycoplasma bovis* was amplified with the specific primers MBOr (5´ CCG TCA AGG TAG CAT CAT TTC CTA T 3´) and MBOf (5´CCT TTT AGA TTG GGA TAG CGG ATG 3´), with 360bp product by Gonzáles et al. (28) and the following cycle profile: one cycle at 94ºC for three minutes, thirty-five cycles at 94ºC for one minute, 60ºC for one minute, 72ºC for one minute and a final step at 72ºC for three minutes.

The amplified material was evaluated according to electrophoretic run in 1.5% agarose gel added of 0.06µL/mL Nancy (sigma). Electrophoresis was run in a horizontal cube containing TBE 1X (89 nM Tris-HCl, 89 mM boric acid and 20 mM EDTA) at a voltage of 65V. The gel was visualized in UV transilluminator and the image was captured by a digital documentation system. From the amplified material, 8µL were used and as molecular weight marker, 4 µL 100pb ladder (Invitrogen) were employed. For all samples, 2 µL running buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 70% Milli-Q water) were added.

RESULTS

Of 185 mastitic secretion, 33 (17.84%) were positive for *Mollicutes*, of which 7 (21.21%) were positive for *Mycoplasma bovis*, in a total of 3.78% cases of mastitis by *M. bovis*.

Of 50 nasal swab samples, 46 (92%) were positive for *Mollicutes*, of which 10 (21.74%) were positive for *Mycoplasma bovis.*

As to clinical signs in calves, 44 (88%) animals had clinical signs of pneumonia. Of which, 7 (15.91%) showed other signs like arthritis and otitis associated with pneumonia. Distribution of the number of mastitic secretion positive for *M. bovis* according to the farms and number of samples was 2 (2.69%), 4 (14.3%) and 1 (12.5%) for farms A, C and D, respectively. Positivity for the pathogen was not detected among the 73 cases of mastitis in farm B. Considering nasal swabs from calves positive for *M. bovis* (n=10), positivity was 9 (40.9%) and 1 (11.1%) for farms A and D, whereas samples from farms B and C were negative for *M. bovis*.

DISCUSSION

Species of the genus *Mycoplasma* are highly contagious (29). In the herd, they disseminate during milking through aerosol and secretions from animals with respiratory and genital disorders. Hematogenic and lymphatic routes are responsible for dissemination from

one organ to the other. Thus, lack of diagnosis may risk the health of the remaining animals, while proper diagnosis of *Mycoplasma* spp. contributes to preventing infection in other animals at the farm (30).

Rapid diagnosis using molecular techniques allows detecting infected animals, preventing thus outbreaks of mastitis and other diseases caused by mycoplasmas. Studies have demonstrated the efficacy of PCR as a diagnostic tool to detect *Mycoplasma* spp. (31).

Prevalence of mastitis caused by *M. bovis* varies from 0.5 to 35% in several countries (32). In Brazil, research is less frequent on this microorganism considering mastitis and pathologies of the respiratory and urogenital systems, articulation, nervous system and conjunctiva ocular (23). In studies conducted by Pretto et al. (19) in Paraná State and southeast region of São Paulo State, the frequency of mastitis by *M. bovis* was 5.83%, a result superior to that found in the present paper, which was 3.78%. However, the present result corroborates those obtained for other countries (32).

Animals detected as positive in the farm should be separated and their milk should not be used since calves could become infected and asymptomatic carriers of mycoplasmas (33). The main control measure against this agent is to limit the entrance of new animals to the dairy farm by applying biosafety measures, including the disposal of positive animals (8,19).

The presence of this microorganism in upper airways does not necessarily mean infection, Nicholas and Ayling (7) but compromises the immune system of the host, Poumarat et al. (34) Gagea et al. (35), allowing invasion of the respiratory system by other pathogenic agents.

All calves positive for *Mycoplasma bovis* had respiratory clinical signs, which suggests that this is the agent responsible for the infection, in spite of the compromising of the immune system of animals, making them more susceptible to infection by other infectious agents (35).

It must be highlighted that 15.91% (7/44) calves positive for *M. bovis* had other clinical signs like arthritis and otitis associated with respiratory disorders, corroborating the participation of this microorganism in different infectious processes due to its lymphohematogenous dissemination (9).

High positivity (92.0%) was found in nasal swabs for the class Mollicutes probably because the primers used for genetic detection of this bacterial class include other species common to the respiratory tract which belong to the genus *Mycoplasma (*14,16).

Analyzing the results found for *Mollicutes*, which were 17.84% positivity with 3.78% in cases of mastitis by *M. bovis*, the participation of other *Mycoplasma* species can be speculated (20,21). Although the number of samples evaluated in farm B was large, no animal positive for the agent was found. Thus, no calf had positivity in the search for *Mollicutes* and *M. bovis* in nasal swabs. This result must be related to the zoosanitary management already established in that farm, considering that the microorganisms are highly contagious, especially *M. bovis.*

As to the examination of 50 nasal swabs, 46 (92%) were positive for *Mollicutes*, of which 10 (21.74%) were positive for *M. bovis*, representing 20% total samples. Farms B and C only had negative samples; concomitantly, only farm B did not reveal the presence of the agent in mastitic milk samples, differently from farm C which, although negative for the agent in nasal swabs, had 14.3% positivity for *M. bovis* in mastitis.

Differences in the results were expected both for cases of mastitis and for nasal swabs from the farms, considering *Mollicutes* and *M. bovis*, which occurred regardless of the number of samples evaluated in each farm. In farm A, which had the greatest number of mastitic secretion samples (n=76) and nasal swabs (n=22), there were 2.6% cases of mastitis and 40.9% respiratory problems caused by *M. bovis* in both sample types. This specific situation made it difficult to evaluate whether the agent was transmitted from the calf to its mother during breast-feeding or not. Such evaluation is only possible if the isolates from the calf and its respective mother with mastitis are characterized.

CONCLUSIONS

Mycoplasma bovis was detected by using PCR technique in nasal swabs from both calves that were healty or had pneumonia and also in mastitic secretion, and confirm the participation of this pathogen in bovine mastitis and that it is part of the pulmonary microbiota of calves. It can plays a significant role in the etiology of bovine mastitis, as well as in pneumonia of calves. The differentiation from other mycoplasma species using specific primers is suggested due to the importance of this group of pathogens in animal pathology.

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