http://dx.doi.org/10.1590/1678-4162-12140


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***Arq. Bras. Med. Vet. Zootec.*, v.73, n.2, p.293-301, 2021**

# Proposal of an iELISA for *Mycoplasma bovis* diagnosis in dairy cattle and associated risk factors

[*Proposta de um iELISA para diagnóstico de* Mycoplasma bovis *em bovinos leiteiros e fatores de risco associados*]

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## ABSTRACT

*Mycoplasma bovis* is a highly contagious agent associated with several pathologies in cattle. The detection of reactive antibodies to *M. bovis* by Indirect Enzyme-Linked Immunosorbent Assay (iELISA) identifies if there was an exposure to the microorganism. The current study aimed to optimize an iELISA from *M. bovis* total cell antigen, applying it to bovine serum samples, and to evaluate risk factors. Serum samples were obtained from 400 cows from 17 herds from Southeast Brazil. In the optimization of iELISA, the following was established: 2 μg/mL of antigen, sera dilution 1:300, and conjugate dilution 1:15000. The frequency was 62.3% (249/400) of reactive animals and 100% (17/17) of reactive herds. Risk factors were: herds with more than 100 animals (OR= 3.1; CI= 95%); Holstein breed (OR= 72.5; CI= 95%); cows (OR= 29.7; CI= 95%); intensive breeding system (OR= 3.3; CI= 95%); associated small ruminant production (OR= 4.4; CI= 95%); milk production above 500L (OR= 2.9; CI= 95%); no quarantine (OR= 1.5; CI= 95%); mechanical milking (OR= 5.5; CI= 95%) and cases of mastitis (OR= 5.5; CI= 95%). The proposed iELISA was able to detect antibodies reactive to *M. bovis* in bovine serum. Knowledge of these risk factors can assist in the implementation of prophylactic measures.

Keywords: mycoplasmosis, mastitis, serology, Holstein breed, cows

## RESUMO

Mycoplasma bovis *é um agente altamente contagioso relacionado a várias patologias em bovinos. A detecção de anticorpos reativos a* M. bovis *por Ensaio de Imunoadsorção Enzimática Indireto (iELISA) identifica se houve exposição ao microrganismo. O presente estudo teve como objetivo otimizar um iELISA de antígeno celular total de* M. bovis*, aplicando-o a amostras de soro bovino, bem como avaliar fatores de risco. Amostras de soro foram obtidas de 400 vacas de 17 rebanhos da Região Sudeste do Brasil. Na otimização do iELISA foram obtidos: 2μg/mL de antígeno, diluição dos soros 1:300 e do conjugado 1:15000. A frequência de animais reativos foi de 62,3% (249/400) e de 100% (17/17) para os rebanhos. Os fatores de risco foram: rebanhos com mais de 100 animais (OR= 3,1; IC= 95%); raça Holandesa (OR= 72,5;IC= 95%); vacas (OR= 29,7;IC= 95%); sistema intensivo (OR= 3,3; C= 95%); produção de pequenos ruminantes (OR= 4,4;IC=95%); produção de leite acima de 500L (OR= 2,9;IC= 95%); sem quarentena (OR= 1,5;IC= 95%); ordenha mecânica (OR= 5,5;IC= 95%) e casos de mastite (OR= 5,5;IC= 95%). O iELISA proposto foi capaz de detectar anticorpos reativos a* M. bovis *no soro bovino. O conhecimento desses fatores de risco pode auxiliar na implementação de medidas profiláticas.*

*Palavras-chave: micoplasmose, mastite, sorologia, raça Holandesa, vacas*

Recebido em 31 de julho de 2020
Aceito em 13 de janeiro de 2021
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## INTRODUCTION

*Mycoplasma* species can cause a variety of diseases in cattle, such as mastitis, otitis media, conjunctivitis, endocarditis, respiratory, joint, brain and reproductive disorders. *Mycoplasma bovis* is highly contagious and the leading agent involved in cases of mastitis in dairy cattle. It can cause outbreaks in the clinical form with a decrease in milk production and quality, leading to the early culling of animals, while in the subclinical form, it is responsible for the maintenance and dissemination of the agent in the herd (Maunsell *et al*., 2011; Fox, 2012; Arede *et al*., 2016; Rosales *et al*., 2017; Kanda *et al*., 2019).

*Mycoplasma bovis* is commonly associated with chronic diseases in cattle. Once established in the herd, it is difficult to eradicate, due both to its invasion of epithelial and immune cells and dissemination to inaccessible parts of the organism, making treatment with antibiotics difficult. Additionally, only a few groups of antimicrobials can be used in its treatment, due to the absence of a cell wall. Furthermore, its widespread use has resulted in observed resistance to these drugs. Moreover, *M. bovis* has great antigenic variation, which hinders the development of vaccines for its control (Pretto *et al*., 2001; Burki *et al*., 2015; Perez-Casal *et al*., 2017).

Acquisition of new, asymptomatic animals, a lack of prior knowledge of their clinical history, increased herd size, as well as agglomeration and confinement are associated with a greater risk of infection by *M. bovis*, which may contribute to the morbidity and mortality of animals (Maunsell *et al*., 2011; Fox, 2012; Aebi *et al*., 2015; Lysnyansky *et al*., 2016; Wawegama *et al*., 2016). Antibody expression against *M. bovis* may persist for several months. Diagnosis by Indirect Enzyme-Linked Immunosorbent Assay (iELISA) detects antibody response, bypassing the need for the active shedding of microorganisms at sample collection. Thus, iELISA is an essential tool for verifying the maintenance of *M. bovis*-free herds, as well as the introduction of newly acquired animals in the herd (Parker *et al*., 2018). According to previous research in Portugal, Brazil, China and Australia, the between-herd prevalence of *M. bovis* by iELISA has been found to vary between 46.7% and 98.0% (Raposo, 2009; Mesquita *et al*., 2015) and within-herd between 3.1% and 98.0% (Raposo, 2009; Fu *et al*., 2014; Mesquita *et al*., 2015; Hazelton *et al*., 2018).

Commercial iELISA kits such as Bio K 260 and Bio K 302 (Bio-X Diagnostics- Rochefort, Belgium) and Bovicheck *M. bovis* (Biovet Inc-Quebec, Canada) are available for the detection of anti- *M. bovis* antibodies in serum, plasma, and bovine milk samples (Parker *et al*., 2018). However, these kits are not yet available in some countries, such as Brazil. Additionally, there is a report on the development of an in-house iELISA for the detection of anti- *M. bovis* antibodies that shows better results when compared to kits (Wawegama *et al*., 2014, 2016). The current study aimed to optimize an iELISA from *M. bovis* total cell antigen, applying it to bovine serum samples, and to evaluate risk factors.

## MATERIALS AND METHODS

This study was approved by the Ethics Committee on the Use of Animals (CEUA) of Universidade Federal Fluminense (UFF), under certificate number 987/2017. *Mycoplasma bovis* Donetta PG 45 from the Mollicutes collection from Universidade de São Paulo (USP) was cultivated in modified Frey medium (Frey *et al*., 1968). Confirmation of growth was checked by the color change in the liquid medium and visualization of typical colonies in the shape of a "fried egg" in a solid medium with a stereomicroscope (Biofocus®, Brazil). The strain was cultivated in increasing volumes up to 1L, at 10% of the culture medium volume. For each batch, a plate was prepared to verify the log growth phase of the strain ("massal" growth). The culture in liquid medium was centrifuged at 4ºC, at 3136 x g for 40 minutes (Hettich Zentrifugen, 420R®, Germany), and the supernatant discarded. The pellet was resuspended in phosphate-buffered saline (PBS) solution pH 7.2 and centrifuged once again as before. The procedure was repeated twice, and the final pellet re-suspended in 2 mL of PBS and kept at -20ºC until protein dosage. The protein concentration was determined (Lowry *et al*., 1951) to detect the concentration of antigen for the sensitization of microtiter plates.

To determine the ideal *M. bovis* antigen concentration, 2, 4 and 8 µg/mL of antigen were tested in the sensitization of microtiter plates (Kartell®, Italy), with the dilution performed in 0.1M carbonate-bicarbonate buffer, pH 9.6. In order to determine the optimal serum dilution, a commercial sterile fetal bovine serum (Sigma Chemical Co. St Louis, MO®, United States) as a negative control and *M. bovis* hyper-immune reactive bovine serum as a positive control were used (Mesquita *et al*., 2015). The tests were performed with the controls distributed in a base 2 serial dilution (ranging from 1/100 to 1/3200) and a base 3 serial dilution (ranging from 1/100 to 1/24300). For the determination of the optimal dilution of the conjugate, rabbit anti-bovine IgG conjugated with peroxidase enzyme (Sigma Chemical Co. St Louis, MO®, United States) was used, and dilutions of 1:10000 and 1:15000 were tested. The chromogenic substrate used was ortho-phenylenediamine (OPD) (Sigma Chemical Co. St Louis, MO®, United States), and the reaction was stopped with 3N sulfuric acid. Reading of optical densities (O.D) was performed with an ELISA reader (DNM-9602, Beijing Perlong New Technologies Ltd.®, China) at a 450 nm wavelength.

Sampling was based on an estimated prevalence of 50% (since *M. bovis* antibody frequency is unknown in the studied region), with a 95% confidence interval and a 5% error, according to the formula $n = Z^2 \times P (1-P) / E^2$ (Thrusfield, 2004), requiring at least 384 animals. In this way, 400 cows were selected from 17 bovine herds from dairy basins in Southeast Brazil as follows: Minas Gerais state (n=147), municipalities of Juiz de Fora (n=68) and Belmiro Braga (n=79); Rio de Janeiro state (n=151), municipalities of Cachoeiras de Macacu (n=44), Duas Barras (n=25), Rio Bonito (n=14), Teresópolis (n=26) and Valença (n=42); and São Paulo state (n=102), municipalities of Areias (n=30), Silveiras (n=33) and São José do Barreiro (n=39). Herds were selected by convenience (non-probabilistic sampling) and animals from each herd were randomly selected.

Based on a cross-sectional study, between March 2018 and July 2019, blood samples were collected from apparently healthy females of reproductive age, from different ages, breeds and stages of lactation, with unknown previous clinical history. Before collection, antisepsis was performed with 70% ethyl alcohol, and then the caudal vein was punctured with a sterile needle and Vacutainer® adapter and packed in a tube without anticoagulant. Meanwhile, a questionnaire to investigate milking management was applied to those responsible for herds for risk factor analysis. Samples were transported under refrigeration, not exceeding four hours from collection, then under laboratory conditions tubes were centrifuged at 591 x g (HT®, Brazil) for 15 minutes to obtain serum, which was transferred to microtubes and stored at -20ºC until iELISA performance.

Samples were tested in triplicate, according to the ideal conditions obtained during the optimization step, with detection of O.D performed as previously described. The reactivity of each sample was calculated by determining the sample/positive serum ratio (S/P), using positive and negative control sera as a reference, where S/P= (sample mean - negative control mean) / (positive control mean - negative control mean). The cutoff value was determined to be two and a half times the mean absorbance of the negative control, and readings above the cutoff were considered reactive (Baldani *et al*., 2004).

For iELISA optimization, Analysis of Variance (ANOVA) was performed with a 95% significance level to determine the ideal concentration of antigen, serum dilution and conjugate dilution. The relative frequency of bovine serum samples reactive to iELISA by state was performed using Pearson's chi-square test with a 95% significance level. For prior analysis of risk factors was performed Pearson's chi-square test or Fisher's exact test with a 90% significance level to prevent possible risk factors from being excluded from the analysis (Murai and Higuchi, 2019). Logistic regression analysis was then performed, with a 95% significance level. The iELISA result was considered a dependent variable, while the independent variables were: herd size (up to 100; more than 100), predominant breed (Holstein; crossbred), age (cows; heifers), rearing system (intensive; semi-intensive; extensive), small ruminant breeding (yes; no), average herd milk production (up to 500L; more than 500L), quarantine in the acquisition of new animals (yes; no), type of milking (mechanical; manual), California Mastitis Test (CMT) application (yes; no) and

occurrence of mastitis in the herd (yes, no). All analyses were performed using BioEstat 5.0 software (Ayres *et al*., 2007).

## RESULTS

Protein dosage of *M. bovis* antigen was 1.8 mg/mL, validated by an $R^2$ value of 0.9956. In order to determine the ideal antigen concentration, the most economical conditions were chosen as follows: base 3 serial dilution of sera and dilution of the conjugate 1:15000, with concentrations of 2, 4, and 8 µg/mL of antigen for sensitizing the plates. There was no difference (p>0.05) for the positive control (p= 0.9536) and the negative control (p=0.5790) in the tested concentrations. The increase in antigen concentration did not imply an increase in the O.D of the positive control. Therefore, a concentration of 2 µg/mL of antigen was chosen to sensitize the microtiter plates, as it was the most economical (Figure 1a).

In the serum dilution test, the plates were sensitized with 2 µg/mL of antigen and dilution of the conjugate 1:15000. There were lower baseline values for the negative controls, as well as better discrimination between the positive and negative controls in the base 3 serial dilution, with the greatest discrimination in the 1:300 dilution (positive control value 8.3 times greater than that of the negative) (Figure 1b). In order to test the conjugate dilutions (1:10000 and 1:15000), the plates were sensitized with 2 µg/mL of antigen and the base 3-diluted sera. There was no difference (p>0.05) between the two conjugate dilutions for the positive (p=0.1119) and negative (p=0.0824) controls. It was possible to verify good discrimination between the positive and negative controls in both dilutions, although dilution 1:15000 was chosen for economic reasons (Figure 1c).

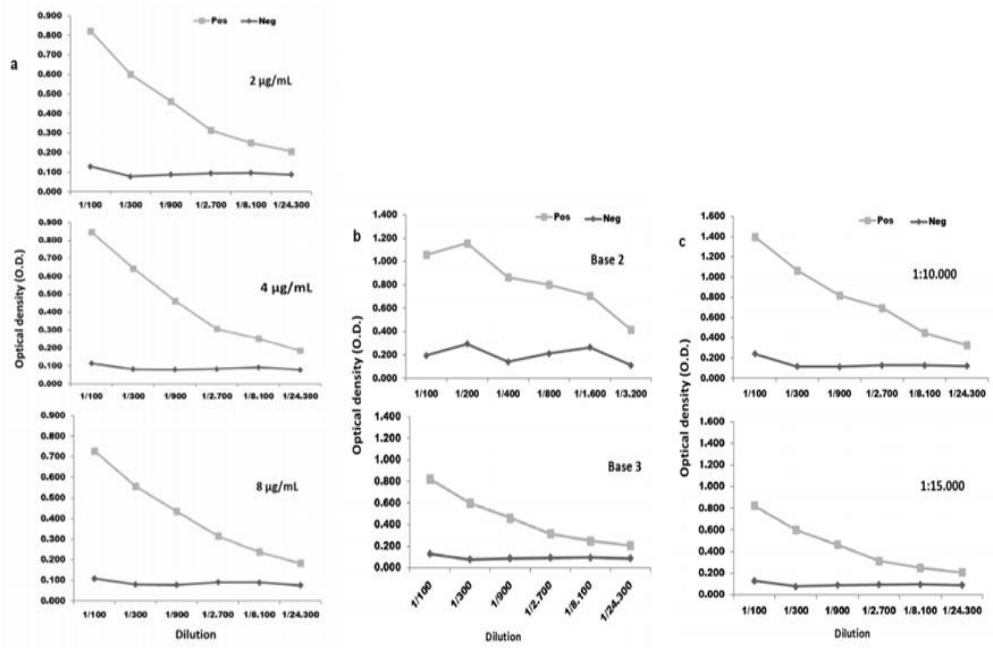


Figure 1. Optimization of iELISA. (a) Determination of the ideal concentration of *Mycoplasma bovis* total cell antigen testing 2, 4 and 8 µg/mL in the sensitization of microtiter plates (p<0.05), with base 3 dilution of sera and dilution of the conjugate 1:15000. (b) Determination of the ideal serum dilution with positive (*M. bovis* hyper-immune reactive bovine serum) and negative (commercial sterile fetal bovine serum) serum control using base 2 and base 3 serial dilution (p<0.05), with a concentration of *M. bovis* antigen of 2 µg/mL and dilution of conjugate at 1:15000. (c) Determination of the ideal conjugate dilution (rabbit anti-bovine IgG conjugated with the enzyme peroxidase) at 1:10000 and 1:15000 (p<0.05), with a concentration of *M. bovis* antigen of 2 µg/mL and base 3 dilution of the sera.

The total frequency of reactive serum animals was 62.3% (249/400), and in 100% (17/17) of the herds there were reactive animals. Concerning the states, there were reactive animals in all municipalities studied (shown in Figure 2), and a greater seropositivity ($p<0.0001$) in São Paulo state (98.1%; 100/102) when compared to Minas Gerais (60.5%; 89/147) and Rio de Janeiro (39.7%; 60/151).

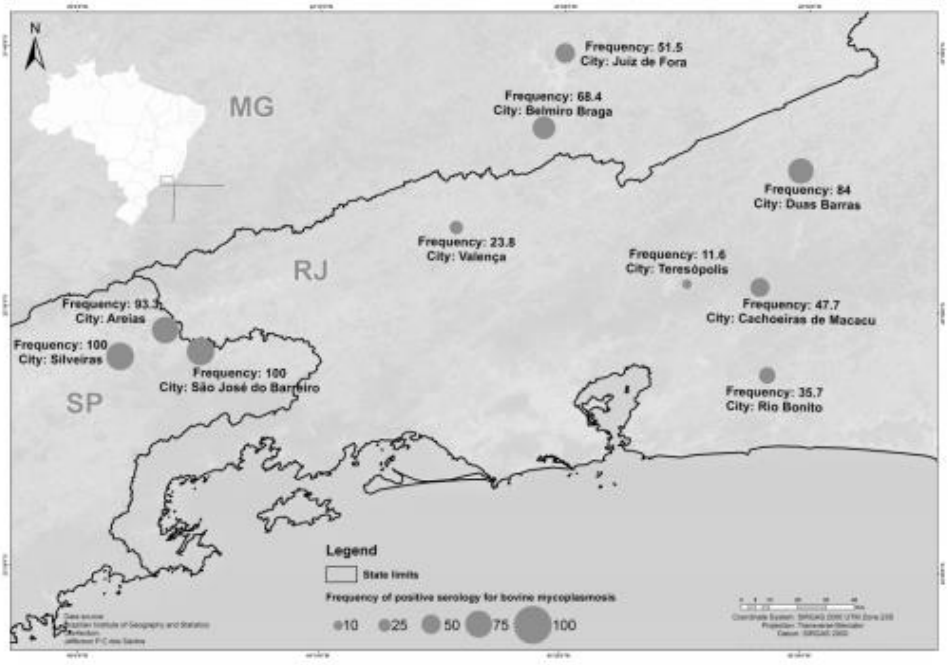


Figure 2. Frequency of bovine serum samples reactive to iELISA *Mycoplasma bovis* total cell antigen from dairy cattle from municipalities of the states of São Paulo, Minas Gerais and Rio de Janeiro, Southeast Brazil, from March 2018 to July 2019.

Risk factors were: herds with more than 100 animals (OR= 3.1, CI= 95%), Holstein breed (OR= 72.5, CI= 95%), age of cows (OR= 29.7, CI= 95%), intensive breeding system (OR=3.3, CI= 95%), small ruminant breeding (OR= 4.4, CI= 95%), milk production greater than 500L (OR= 2.9, CI= 95%), no quarantine (OR= 1.5, CI= 95%), mechanical milking (OR=5.5, CI= 95%) and cases of mastitis in the herd (OR=5.5, CI= 95%), according to Table 1.

## DISCUSSION

In the current study the ideal antigen concentration for sensitizing microtiter plates was the same found by Mesquita *et al*. (2015), who did not evaluate the dilutions of serum and conjugate, but instead kept the base 2 dilution of serum and conjugate at 1:10000. The frequency of reactive animals (62.3%) and herds (100%) here was higher than observed in the later study (3.1% and 46.7%, respectively), which may have been due to the difference in the studied regions. It is also likely that the optimization of sera and conjugate dilution conditions in this study produced an iELISA more sensitive in its detection of reactive serum to the total antigen of *M. bovis*.

Our results were compatible with the detection of *M. bovis* worldwide. In Europe, Raposo (2009) detected serum reactivity of 98.0% for both herds and animals in Portugal and Fodor *et al*. (2017) reported a frequency of 82.9% for animals and 100% for herds in Hungary. Fu *et al*. (2014) reported a variation of 43.9% to 75.8% in seroreactive animals from China and 93.8% of cattle in Australia were also reactive to *M. bovis* (Hazelton *et al*., 2018), however in both studies the animals had clinical manifestations, which did not occur in the present study where the previous clinical history of each animal was unknown.

Table 1. Risk factors associated with bovine seroreactivity in iELISA *Mycoplasma bovis* total cell antigen in dairy cattle from municipalities from the states of São Paulo, Minas Gerais and Rio de Janeiro, Southeast Brazil, from March 2018 to July 2019

| Variable | Category | N | Reactive by iELISA (n/%) | p- value | Logistic Regression OR (CI 95 %)[1] | p- value |
|---|---|---|---|---|---|---|
| Herd size | Up to 100 | 135 | 72 (53.3) | 0.0118(A) | 3.1 (1.4-6.8) | 0.0059(C) |
| | More than 100 | 265 | 177 (66.8) | | | |
| Predominant breed | Holstein | 59 | 53 (89.8) | 0(B) | 72.5(6.4-817.1) | 0.0005(C) |
| | Crossbred | 341 | 196 (57.5) | | | |
| Age | Cows | 290 | 204 (70.3) | < 0.0001(A) | 29.7(11.1-79.3) | <0.0001(C) |
| | Heifers | 110 | 45 (40.9) | | | |
| Rearing system | Intensive | 50 | 42 (84.0) | <0.0001(A) | 3.3 (1.6-7.1) | 0.0013(D) |
| | Semi-intensive | 320 | 198 (61.9) | | | |
| | Extensive | 30 | 09 (30.0) | | | |
| Small ruminant breeding | Yes | 74 | 58 (78.4) | 0.0024(A) | 4.4 (1.9-10.6) | 0.0008(C) |
| | No | 326 | 191 (58.6) | | | |
| Average herd production | Up to 500L | 208 | 106 (51.0) | < 0.0001(A) | 2.9 (1.4-6.4) | 0.0059(C) |
| | More than 500L | 192 | 143 (74.5) | | | |
| Quarantine | No | 257 | 170 (66.1) | 0.0405(A) | 1.5 (1.0-2.4) | 0.0316(D) |
| | Yes | 143 | 79 (55.2) | | | |
| Type of milking | Mechanical | 384 | 243 (63.3) | 0.0618(B) | 5.5 (1.6-19.0) | 0.0064(C) |
| | Manual | 16 | 06 (37.5) | | | |
| CMT[2] application | No | 289 | 178 (61.6) | 0.7466(A) | - | - |
| | Yes | 111 | 71 (64.0) | | | |
| Cases of mastitis in the herd | Yes | 336 | 219 (65.2) | 0.0086(A) | 5.5 (1.5-19.3) | 0.0086(C) |
| | No | 64 | 30 (46.9) | | | |

(A) Associations assessed by Pearson's Chi-square (p<0.10); (B) Fisher's Exact test (p<0.10).
(C)Associations assessed by multiple logistic regression (p<0.05); (D) Associations assessed by simple logistic regression (p <0.05). [1]Odds ratio (Confidence Interval); [2] California Mastitis Test.

*Mycoplasma bovis* is primarily an inhabitant of respiratory mucosal surfaces, often found in the upper respiratory tract of healthy cattle and may cause infection in immunosuppressed animals. Transmission between different body sites in the same animal can occur by lymphatic and blood routes, and intramammary and respiratory infection induce active humoral responses (Maunsell *et al*., 2011; Fox, 2012). *Mycoplasma bovis* generally causes chronic infection due to great antigenic variation, allowing it to escape from the host's immune system. The invasion of epithelial and immune cells allows its dissemination to sites of difficult access in the organism, which may cause suppression of the host's responses and the formation of biofilm that guarantees its permanence in the host (Burki *et al*., 2015; Perez-Casal *et al*., 2017). Chronic infections are more easily detected by iELISA than by Polimerase Chain Reaction (PCR) and culture, which explains the high infection rates detected in serological analysis (Parker *et al*., 2018).

Regarding risk factors, infection with *M. bovis* was about three times greater in herds with more than 100 animals and in those with an average production of more than 500L. Such results corroborate Fox (2012), who reports a greater infection by *M. bovis* in larger herds with greater production, which occurs due to the greater circulation (buying and selling) of often asymptomatic carriers of *M. bovis* (Maunsell *et al*., 2011; Pardon *et al*., 2020).

Holstein breed was the greatest risk factor, more than 70 times greater when compared to crossbred cows. It is already known that one of

the main factors related to low productivity, fertility and health in Holstein cattle, managed under tropical conditions, is heat stress and that crossbreed animals have shown greater environmental adaptability (Khan *et al*., 2018; Gernand *et al*., 2019). Additionally, purebred animals are often kept under intensive breeding systems, which was a risk factor when individually analyzed by simple logistic regression, with a risk of infection about three times greater than in animals raised under other breeding systems. Similarly, Wawegama *et al*. (2016) detected greater antibody response for *M. bovis* in beef cattle kept under confinement in Australia.

Risk in cows was almost 30 times higher when compared to heifers, corroborating Aebi *et al*. (2015), who found that the number of lactations is a risk factor. Also, in herds where cases of mastitis were reported there was a risk more than five times higher than in herds with no cases of clinical or subclinical mastitis. It has been suggested that the production of antibodies reactive to *M. bovis* is due to infection of the mammary glands with or without a manifestation of clinical signs (Parker *et al*., 2018).

The risk of infection when performing mechanical milking was 5.5 times greater than when performing manual milking. Mastitis caused by *Mycoplasma* spp. is contagious, in which the pathogen infects the udder, and is transmitted horizontally during milking through fomites and milkers' hands (Fox, 2012). In this case, the greatest risk of transmission occurred through the use of a mechanical milking machine, corroborating Aebi *et al*. (2015), who found a greater risk of infection with a specific brand of milking equipment. Therefore, authors reiterate the importance of hygiene measures such as hot water, alkaline and acid detergent in utensils and equipment used in mechanized milking, as well as respecting the milking line order (heifers, younger and older cows without mastitis history and cows with mastitis history) avoiding cross-infection among animals.

When analyzed by multiple logistic regression, lack of quarantine was not a risk factor. However, when analyzed by simple logistic regression, the risk of infection in unquarantined animals was 1.5 greater than in herds where isolation protocols were adopted before the introduction of newly acquired animals. In this way, corroborating the work of Murai and Higuchi (2019) and Pardon *et al*. (2020) the lack of a quarantine protocol is a risk factor for *M. bovis* infection.

Concerning contact between animals, the production of other ruminant species such as goats and sheep was also a risk factor for the detection of antibodies reactive to *M. bovis* in iELISA. There may be a cross-reaction with *M. agalactiae*, which affects small ruminants, and in some cases, can infect cows, showing a 99.8% genetic similarity to *M. bovis* (Nicholas *et al*., 2008). As a result, efforts to serologically differentiate both species by means of an iELISA based on recombinant proteins have been unsuccessful (Sun *et al*., 2014; Cheema *et al*., 2016). Due to such serological limitations, molecular techniques have been proposed to differentiate them with a greater degree of reliability (Foddai *et al*., 2005; Wisselink *et al*., 2019).

## CONCLUSIONS

The iELISA proposed was able to detect antibodies reactive to *M. bovis* in bovine serum, in which the high frequency of reactive animals indicates mycoplasma circulation in dairy cattle from Southeast Brazil. Knowledge of these risk factors can assist in the implementation of prophylactic measures aimed at controlling the agent in dairy cattle breeding systems. The risk factors can be considered in regions and herds with similar characteristics to those of the current study.

## ACKNOWLEDGMENTS

The authors would like to thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for the financial support for conducting the research as well as those responsible for the herds, the collection of animal samples and answering questionnaires.

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