



# Prevalence of three *Mycoplasma* sp. by multiplex PCR in cattle with and without respiratory disease in central Mexico

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## Abstract

This study aimed to identify *Mycoplasma bovis*, *Myc. dispar*, and *Myc. bovirhinis*, which are involved in bovine respiratory disease through a multiplex PCR as an alternative to culture's features that hamper *Mycoplasma* isolation. Nasal swabs were taken from 335 cattle with and without respiratory disease background (RDB) from dairy herds in the central region of Mexico. Each sample was divided in two; the first part was processed for the direct DNA extraction of the nasal swab and the second for *Mycoplasma* isolation, culture, and then the multiplex PCR was performed. In the nasal swabs, *Myc. bovis* was identified in 21.1%; *Myc. dispar*, in 11.8%; and *Myc. bovirhinis*, in 10.8% in cattle with RDB. Isolates were identified as *Myc. bovis*, 20.1%; *Myc. dispar*, 11.8%; and *Myc. bovirhinis*, 6.1%. There is a strong correlation between the presence of *Mycoplasma* identified by PCR and the clinical history of the disease ( $\rho < 0.0000$ ). In animals without RDB, *Myc. bovirhinis* was the only species detected in 6.1% of the samples processed directly for multiplex PCR, and in 2% of the isolates. There is an excellent correlation (kappa 0.803) between the isolation and the 16S PCR and a high correlation (kappa 0.75) between the isolation and the multiplex PCR. Therefore, we conclude that the PCR multiplex test is highly sensitive and may be used for the diagnosis and surveillance of the three species in biological samples and mycoplasma isolates.

**Keywords** Cattle · Bovine respiratory disease · *Mycoplasma* · *Myc. bovis* · *Myc. dispar* · *Myc. bovirhinis*

## Introduction

Bovine respiratory disease (BRD) causes estimated losses between \$800 million and \$900 million annually due to death, reduced feed efficiency, and treatment costs (Brooks et al. 2011). These losses result from the generalized state of immunosuppression of the animal, favoured by the stress during handling together with environmental factors that contribute to the establishment of its multifactorial

aetiology (Fulton et al. 2022; Gaudino et al. 2022). The viruses mainly associated with the BRD are bovine herpesvirus-1, the cause of the infectious bovine rhinotracheitis (IBR) (Jones and Chowdhury, 2007, 2010); bovine parainfluenza virus-3 (BPV-3) (Bryson et al., 1979; Mehinagic et al. 2019); bovine viral diarrhoea virus 1 and 2 (BVDV-1–2) (Burciaga-Robles et al. 2010; Goto et al. 2021); bovine respiratory syncytial virus (BRSV) (Fulton et al. 2000; Sorden et al. 2000; Makoschey and Berge 2021); bovine adenovirus A–D (BAV-A–D) (Fent et al 2002; Fulton, 2009) and bovine coronavirus (BCoV) (Storz et al. 1996; Frucchi et al 2022; Soules et al., 2022). The associated bacteria belong to the Pasteurellaceae Family *Mannheimia haemolytica* (Rice et al. 2007; Confer and Ayalew. 2018; Frucchi et al 2022), *Pasteurella multocida* (DeRosa et al. 2000; Storz et al. 2000; Frucchi et al 2022), *Histophilus somni* (Corbeil 2007; Shirbroun 2020), and the genus *Mycoplasma*: *Myc. bovis*, *Myc. dispar* and *Myc. mycoides* subsp. capri. Additionally, *Myc. bovirhinis*, a species that has been isolated from the nasopharyngeal cavity of healthy and diseased cattle, currently has been recognized as a species associated with BRD (Gourlay

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et al. 1979; Rosengarten and Citti, 1999; Angen et al. 2009; Bednarek et al. 2012).

*Mycoplasma* participates in host immunosuppression during the development of BRD (Simmons and Dybvig 2007; Maina et al., 2019) and causes acute clinical symptoms during viral coinfection. *Mycoplasma* is difficult to eliminate in herds, due to its ability to evade the defence mechanisms of the host's immune system such as its surface variable lipoproteins (Christodoulides, et al. 2018), its ability to form biofilms (McAuliffe, et al. 2006) and adhesins that allow it to bind to neutrophils and lymphocytes (Askar, et al. 2021; Yiwen, et al. 2021). These characteristics, together with inadequate treatment and stress, perpetuate its persistence and spread in the cattle herd.

In dairy herds, the first 45 days of life are crucial in breeding cattle since in this period, the largest number of animals dies due to respiratory problems. Therefore, preventive measures such as the isolation of the animals and the use of vaccines are implemented to mitigate the economic impact of BRD (Loneragan et al. 2001; Dubrovsky et al. 2019). Hence, it is necessary to identify the causal agents to carry out the corrective biosecurity measures and the appropriate treatments for their control.

This study aimed to identify *Myc. bovis*, *Myc. dispar* and *Myc. bovirhinis* through their isolation and the application of a multiplex PCR in cattle with and without respiratory disease background to validate its use as a rapid diagnostic method allowing the reduction of the diagnosis time through culture.

## Material and methods

### Animals

A random sampling of cattle under 1 year of age was carried out in dairy herds of the central area of Mexico: Hidalgo, Aguascalientes, Guanajuato and Puebla. We considered clinically healthy animals that had not shown clinical symptoms of respiratory disease at the moment of the sampling, and according to the herd's records, they had no respiratory disease reports in their lifetime. Animals considered to have respiratory disease were those that at the time of sampling had nasal discharges, cough, fever and/or pneumonia.

Each swab was stored in a sterile modified Hayflick medium (Jasper, 1981), transported to the laboratory at 4 °C, and then divided into two aliquots. The first one was for DNA extraction and the second for bacterial isolation. In compliance with the formula  $n = Z^2 p.q/d^2$ , the sample size was 335 cattle, from which 194 (57.91%) were sick animals with respiratory disease and 141 (42.08%) healthy animals.

### Bacterial isolation

The *Mycoplasma* isolation was performed as stated by the methodology of Tully (1983); each sample was inoculated into modified Hayflick media, resuspended and divided into two aliquots. The first was used for DNA extraction directly from the nasal swab, and the second was serially diluted into a liquid medium from  $10^{-1}$  to  $10^{-3}$  for mycoplasma isolation. Each dilution was spread in a solid medium and further incubated at 37 °C in microaerophilic conditions in a candle jar. The pH changes, indicated by phenol-red, and/or slight turbidity were periodically examined as indicative of the bacteria development. Single *Mycoplasma* colonies were excised within tiny sections of agar, and they were inoculated into liquid media. The reseeded process was repeated at least three times until colonies had the typical fried egg appearance in a solid medium. Each *Mycoplasma* culture was assessed by 0.45 and 0.22 µm filtration, non-reversion to L-forms and digitonin sensitivity (Tully 1983; Howard and Rosenbusch, 1994).

### DNA extraction

DNA was extracted from the nasal swabs by the guanidine thiocyanate protocol (Green and Sambrook, 2012). *Mycoplasma* has a long replication time, small size and small genome. Therefore, in order to harvest enough DNA, each *Mycoplasma* isolate was scaled up to 50 ml in Hayflick medium as follows. The first homogenization was performed by shaking the swab into 2 ml of Hayflick medium, from which 200 µl was added to 1.8 ml of fresh medium, and seeded in solid media to obtain single colony isolates. Then, each isolation was seeded in a fresh liquid medium, and as soon as the colour change of the prime culture was observed, approximately 12 h later, the prime culture was enriched with twice its volume to promote the development of *Mycoplasma*. This procedure was repeated until the required volume was obtained and harvested by centrifugation at  $11,300 \times g$  for 45 min at 4 °C, washed in triplicate with PBS (7.2 pH) and kept frozen at -20 °C until the DNA extraction by the above-mentioned protocol. In our experience, a minimum of 20 ml of culture is sufficient for DNA extraction and will take about 15 to 20 days of incubation.

### Endpoint PCR and multiplex PCR

The genus-specific PCR based on the 16S rRNA, with the primers forward 5'-CTGGCTGTGTGCCTAATACATGC-3' and reverse 5'-CTTCGGGCATTACCAGCTCC-3', was applied (L'Abée-Lund et al. 2003) with the following conditions: 96 °C for 5 min, followed by 30 cycles of 94 °C

for 45 s, 68 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The amplified product was 1370 bp. *Myc. bovis* DNA was used as positive control and *Mannheimia haemolytica* DNA as a negative control. The nasal swab samples and the isolates that were 16S rRNA PCR positive were subsequently assessed by multiplex PCR.

The multiplex PCR was based on the previously reported amplification of *oppD* and *oppF* from *Myc. bovis* (Hotzel et al. 1996), the *rpoB* gene for *Myc. dispar* identification (Rojas-Trejo, 2013) and new primers based on the *rpoB* gene of *Myc. bovirhinis*, GenBank accession number FJ765326.1 (Table 1). Reference strains of *Myc. bovis* (strain Donetta, ATCC 25,523), *Myc. dispar* (ATCC 27,140) and *Myc. bovirhinis* (ATCC 27,748) were used as controls. The reaction was prepared in 25 µl volume: 7.5 µl of H<sub>2</sub>O, 2.5 µl of 10× buffer, 3.5 mM MgCl<sub>2</sub>, 200 mM dNTP, 10 µl DNA, 20 mM of each primer and 1.25 U Taq polymerase. The amplification program was 95 °C for 5 min, followed by 33 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. PCR products were visualized by electrophoresis at 75 V for 55 min on a 1.5% agarose gel and stained with 0.5 mg/ml of ethidium bromide. DNA bands were visualized on a UV transilluminator and documented with a Gel Logic 212 Pro (Carestream, Woodbridge, CT, USA). As a molecular weight marker, a lane with 1 kb plus (Invitrogen) was included.

## Statistical analysis

The non-parametric statistical analysis of chi-square was applied with 95% confidence ( $p = 0.005$ ), with Statistical Package for the Social Sciences (SPSS), Windows, version 20.0. The accuracy measures for the multiplex PCR and *Mycoplasma* isolation were calculated, and the agreement measures between the multiplex PCR and isolation results were estimated by the Cohen's kappa statistic (Cohen, 1960).

## Results

The isolation of *Mycoplasma* spp. was possible in eighty-seven (25.97%) of the 335 nasal swabs; nine (10.34%) from healthy cattle, and seventy-eight (89.65%) from cattle with respiratory disease background. In three (3.4%) nasal swabs, more than one colony morphology was observed. All the isolates were bacteriologically typified as *Mycoplasma* spp. as they were digitonin sensitive, 0.45 and 0.22 µm filterable, retained their form and were positive to the genus-specific PCR based on the 16S rRNA (Supplementary Fig. S1; Diagram S1).

The PCR based on the 16S rRNA identified 115 (34.32%) positive nasal swabs; 99 (51.03%) from the 194 cattle with respiratory disease background and 16 (11.34%) from the 141 healthy cattle (Diagram S1). When the multiplex PCR was applied to the 99 nasal swabs from cattle with respiratory disease background, 79 were positive and 20 were negative to the species identified by this molecular tool remaining as *Mycoplasma* spp. in the light of the genus-specific PCR. When the multiplex PCR was applied to the 16 nasal swabs from healthy cattle, seven were positive and nine were negative, and also remained as *Mycoplasma* spp. (Table S1; Table 3; Diagram S1). These results mean that only 86 (74.78%) out of the 115 nasal swabs, previously identified by the 16S-PCR, were positive when the multiplex PCR was applied. These 86 positive nasal swabs to the multiplex PCR yielded 101 individual results as follows: *Myc. bovis* 43 (42.57%), *Myc. bovirhinis* 35 (34.65%) and *Myc. dispar* 23 (22.77%).

Regarding the isolation, from the eighty-seven samples that showed bacterial growth, fifteen samples (17.24%) had more than one result for the mycoplasma species identified by the multiplex PCR, from which *Myc. bovis* was the most frequently isolated (Table 2; Tables S1, S2; Diagram S1). Twenty-nine samples that previously were positive for the 16S-PCR were negative for the mycoplasma species identified by the multiplex PCR, and 43 remained positive for only one mycoplasma species.

**Table 1** Multiplex PCR primers for the *Mycoplasma* species

| <i>Mycoplasma</i> species | Primer sequences (5'-3')   | Product size (bp) | Reference            |
|---------------------------|--|-------------------|----------------------|
| <i>Myc. bovis</i>         | GCTCTTTTTGAACAAATACGTCAAGAG<br>GGCTCTCATTAAGAATGTCTTTGTCTAA      | 1907              | Hotzel et al. (1996) |
| <i>Myc. dispar</i>        | GAGTACTTCTCTGGTCCTGGAGGTC<br>GCGGGCAATATCAGATTCAATCCAGTTG        | 548               | Rojas-Trejo (2013)   |
| <i>Myc. bovirhinis</i>    | TGGATCAAATTAATCCATTATCAGAAGTTTC<br>GTTCCGTTAGTATAAGTATAGTTACGACG | 397               | This work            |

bp base pair

**Table 2** Comparison between the results of the multiplex PCR performed in the complete nasal swab samples and in the individual *Mycoplasma* colonies

| <i>Mycoplasma</i> species | <i>Myc. bovis</i> | <i>Myc. dispar</i> | <i>Myc. bovirhinis</i> | Positive Nasal swab samples   | Individual <i>Mycoplasma</i> colony |
|---------------------------|-------------------|--------------------|------------------------|-------------------------------|-------------------------------------|
| <i>Myc. bovis</i>         | 8                 | 3                  |                        | 43 - 11 double infection = 32 | 40                                  |
| <i>Myc. bovirhinis</i>    |                   |                    |                        | 35 - 7 double infection = 28  | 20                                  |
| <i>Myc. dispar</i>        |                   | 4                  |                        | 23 - 12 double infection = 11 | 23                                  |
| <i>Mycoplasma spp</i>     |                   |                    |                        |                               | 16                                  |
| <b>TOTAL</b>              | 86                |                    |                        |                               | 99                                  |

**Table 3** Comparison between the results of the multiplex PCR performed in healthy cattle and cattle with diseased backgrounds and the isolation results within both groups

| <i>Mycoplasma</i> species | Healthy cattle (total 141)<br>16 nasal swabs were 16S-PCR positive |                                     | Cattle with disease background (total 194)<br>99 nasal swabs were 16S-PCR positive and 78 were positive for isolation |                                     |
|---------------------------|--|-------------------------------------|---|-------------------------------------|
|                           | Multiplex PCR Nasal swab   | <i>Mycoplasma</i> individual colony | Multiplex PCR Nasal swab  | <i>Mycoplasma</i> individual colony |
| <i>Myc. bovis</i>         | –  | –                                   | 43  | 40                                  |
| <i>Myc. bovirhinis</i>    | 7  | 4                                   | 28  | 16                                  |
| <i>Myc. dispar</i>        | –  | –                                   | 23  | 23                                  |
| <i>Mycoplasma spp.</i>    | –  | 5                                   | 21 <sup>c</sup>   | 11                                  |
| <b>Total</b>              | 7 <sup>a</sup>   | 9                                   | 78 <sup>b</sup>   | 90                                  |

<sup>a</sup>3 of them had no isolation

<sup>b</sup>15 had more than one result

<sup>c</sup>Nasal swabs that were 16S-PCR positive and negative to the multiplex PCR

The multiplex PCR discriminates among the three mycoplasma species based on the length of the amplified products: 1907 bp (*Myc. bovis*), 548 bp (*Myc. dispar*) and 397 bp (*Myc. bovirhinis*) as shown in Supplementary Fig. 2. The limit of detection was calculated based on the genome sequences of each species and established at 2568 genome equivalents (GE) for the *Myc. dispar* PCR, at 6737 GE for the *Myc. bovirhinis* PCR and at 6730 GE for the *Myc. bovis* PCR. For the multiplex PCR, we used a mixture of the three DNAs to establish the limit of detection at 3516 GE. The estimation of GE was based on the 1,003,404 bp *Myc. bovis*

ATCC 25,523 genome (Wise et al. 2011), the 1,065,810 bp *Myc. dispar* GS01 strain genome (Chen et al. 2019), the 948,039 bp *Myc. bovirhinis* strain HAZ141\_2 genome (Hata et al. 2017) and the average molecular weight of a base pair of 617.96 g/mol.

Ninety-nine individual *Mycoplasma* colonies were retrieved from the 87 nasal swabs that were positive for the isolation. All of them were assessed by the multiplex PCR and identified as *Myc. bovis* 40 (40.40%), *Myc. bovirhinis* 20 (20.20%) and *Myc. dispar* 23 (23.23%) and sixteen colonies (16.16%) as *Mycoplasma spp.* (Table 2; Diagram S1).

The comparison between the isolation-positive nasal swabs from healthy cattle and cattle with disease backgrounds shows that in the nine healthy cattle, only four were identified by the multiplex PCR and five remained as *Mycoplasma* spp. in the light of the 16S-PCR. Aside from the seventy-eight isolation-positive nasal swabs from cattle with disease background, sixty-six were identified by the multiplex PCR with multiple results, and twelve remained positive to only *Mycoplasma* spp. in the light of the 16S-PCR (Table S1; Table 3; Diagram S1).

When comparing the individual colonies' results with the multiplex PCR results, only four colonies out of nine isolated from healthy cattle were identified by the multiplex PCR as *Myc. bovirhinis* and five remain as *Mycoplasma* spp. Regarding the ninety individual colonies retrieved from cattle with disease backgrounds, 40 were identified as *Myc. bovis*, 23 as *Myc. dispar*, 16 as *Myc. bovirhinis* and 11 remained as *Mycoplasma* spp. (Table 3; Diagram S1). When *Mycoplasma* isolation is regarded as the gold standard, the kappa statistic (kappa 0.803) between the isolation and the 16S PCR was interpreted with "excellent agreement" (Fleiss et al., 2003) and that between isolation and the multiplex PCR was a high correlation (kappa 0.75). Regarding the measures of accuracy between the multiplex PCR results and isolation, based on the species level, the multiplex PCR showed 82% sensitivity and 46% specificity.

The prevalence of the *Mycoplasma* genus in dairy herds in the central region was 34.32%. There is a positive correlation between the previous background of respiratory disease and the presence of *Mycoplasma* identified by PCR in nasal swabs  $\rho < 0.0000$ . Additionally, *Myc. bovirhinis* was the most significant of the three species  $\rho = 0.005$  ( $< 0.05$ ) in healthy cattle and *Myc. bovis* in cattle with disease background. Both species were successfully identified by the multiplex PCR.

## Discussion

The BRD at necropsy is associated with caseous bronchopneumonia and coagulative necrosis, with poor response to antibiotic treatment in chronic pneumonia, and the main species associated with this presentation is *Myc. bovis*. (Gagea et al. 2006; Caswell and Archambault 2007; Kleinschmidt et al. 2013). *Myc. dispar* has been reported mostly in adults, and it causes pneumonic lesions (Pirie and Allan 1975; Gourlay and Howard 1982), and in young animals, it causes immunosuppression. In this study, we identified *Myc. dispar* in cattle between ages of 180 and 225 days. Given that *Myc. bovirhinis* is part of the normal microbiota of cattle (Gourlay et al. 1979; Allen et al. 1992), we can explain why we identified this mycoplasma species in only 6.1% of healthy animals; these cattle were also identified as negative by the

multiplex PCR, probably caused by a low yield of DNA during the extraction that may have been under the PCR limit of detection. On the other hand, *Myc. bovis* has been also identified in asymptomatic cattle and as the cause of mild pneumonia (Prysljak et al. 2011; Franca Dias de Oliveira et al. 2016; Murray et al. 2017). However, when there is the coinfection of bacteria and viruses, it will cause severe disease (Tegtmeier et al. 1999; Angen et al. 2009).

This genus has the feature of lacking a cell wall; it may survive in the environment for several days at temperatures up to 4 °C (Pfutzner, 1984) and perpetuates itself to take part in long-lasting mastitis, arthritis and otitis. These genus features explain why *Myc. bovis*, *Myc. dispar* and *Myc. bovirhinis* are species with a high incidence of up to 50% in the cattle herd in the country centre and the correlation between their identification by PCR and the respiratory disease background in the assessed cattle.

The isolation of a microorganism is subjected to its requirements, the bacterial load and other factors including the presence of inhibitory substances in the sample such as enzymes, antibodies and/or antibiotics, the disease stage and culture conditions; therefore, it is understandable the greater efficiency of PCR for the identification of the *Mycoplasma* genus. As for other mycoplasma species, the application of molecular techniques has more accuracy and sensitivity than culture (Stellrecht et al. 2004; Garcia et al. 2005; Hong et al. 2011; Jamalizadeh Bahaabadi et al. 2014). The multiplex PCR herein applied has a detection limit of 3515 GE. This limit follows previous reports of other applications for *Mycoplasma* spp. which are above 1000 GE (Stemke et al. 1994; Strait et al. 2008; Charlebois et al. 2014), or between  $10^3$  and  $10^6$  colony forming units (Razin, 1994). Additionally, results can be obtained in less than a week compared to the culture which can take 30 days.

The species-specific fragments amplified by the multiplex PCR were easily distinguished on the electrophoresis gel, showing their capacity for the identification with the minimum concentration of genomic DNA of the different species. In this study, *Myc. dispar* and *Myc. bovirhinis* were identified based on the *rpoB* gene. As confirmation, the products of the hypervariable region of the *rpoB* gene of some isolates were sequenced, which allows us to guarantee the specificity of the assay (Das et al. 2014).

Among the limitations of the study, the mycoplasmas that were not identified by multiplex PCR had to be preserved for later studies. Likewise, conventional PCR was chosen over qPCR because there are many places in our country that do not have access to sophisticated laboratories. In those locations, producers and field veterinarians have easier, faster and cheaper access to small laboratories with conventional equipment that may make this test available and affordable.

To the best of our knowledge, mucus does not have inhibitors in great amounts (Schrader et al. 2012), and the



guanidine thiocyanate DNA extraction protocol removes some inhibitory substances, including mycoplasma growth inhibitors. Therefore, the inclusion of internal control was not considered since it must be included in the same reaction and multiplexed at the same time as the multiplex PCR, and a serious drawback is that it can cause the reaction to fail or give a false-positive result. Instead, external positive control, negative control and non-template control were included.

Altogether, the results herein presented showed that the multiplex PCR compared to *Mycoplasma* isolation was highly sensitive (82%), and it can be used as a diagnostic test and for the surveillance of the disease allowing the simultaneous identification of *Myc. bovis*, *Myc. dispar* and *Myc. Bovirhinis*.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11250-022-03398-y>.

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**Author contribution** LMMR performed the material preparation, experiments, and fieldwork. VT contributed to the design and implementation of the research. EMCC provided critical feedback; did data analysis and wrote the manuscript. FTT and REMM designed and directed the research and provided critical feedback. All authors read and approved the final manuscript.

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**Data availability** Supplementary tables are included.

**Code availability** Not applicable.

## Declarations

**Ethics approval** This study was performed with the consent of all farmers. All handling of animals in connection with sampling was performed, considering animal welfare and following international and national guidelines.

**Consent to participate** Verbal informed consent was obtained prior to the interview of the dairy farmers.

**Consent for publication** The interviewed dairy farmers have consented to the submission of the results of this study to the journal.

**Conflict of interest** The authors declare no competing interests.

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