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BRIEF REPORT

Detection by multiplex PCR of *Mycoplasma* species associated with dairy cattle in Argentina

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Abstract There is scarce information about the frequency and epidemiological and clinical features associated with the presence of *Mycoplasma* spp. in Argentine dairy herds. The objectives of this study were to develop a multiplex PCR for identifying *M. bovis* and *M. canadense* and to describe the frequency of *Mycoplasma* spp. isolated from clinical samples submitted to a diagnostic laboratory. Of a total of 1548 samples from intramammary infections, bulk tank milk and biological fluids, 38 *Mycoplasma* isolates were obtained. *M. bovis*, *M. canadense*, *M. californicum* and *M. leachii* were detected by using two multiplex PCRs, confirming their presence in clinical conditions in dairy cattle. The techniques used in the present study can be useful to broaden the knowledge about *Mycoplasma* infections in cattle, since the search for these organisms is not usually included in routine diagnoses.

PALABRAS CLAVE
Ganado lechero;
Mycoplasma spp.;
PCR multiplex

Detección por PCR multiplex de especies de *Mycoplasma* asociadas a ganado lechero en Argentina

Resumen Existe poca información sobre la frecuencia, así como las características epidemiológicas y clínicas asociadas con la presencia de *Mycoplasma* en los rodeos lecheros argentinos. Los objetivos de este estudio fueron desarrollar una PCR multiplex para identificar *M. bovis* y *M. canadense* y describir la frecuencia de especies de *Mycoplasma* aisladas de muestras clínicas enviadas a un laboratorio de diagnóstico. De un total de 1.548 muestras de infecciones intramamarias, leche de tanque de frío y fluidos biológicos, se obtuvieron 38 aislamientos de

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Mycoplasma. Mediante 2 PCR multiplex se detectaron *M. bovis*, *M. canadense*, *M. californicum* y *M. leachii*, confirmando su presencia en síndromes clínicos en ganado lechero. Las técnicas utilizadas en el presente estudio pueden ser útiles para ampliar el conocimiento sobre las infecciones por *Mycoplasma* en bovinos, ya que la búsqueda de estos organismos no suele incluirse en los diagnósticos de rutina.

Mycoplasmas are common inhabitants of the respiratory mucosa, the urogenital tract, conjunctival surfaces, the gastrointestinal tract and the mammary gland; they can be commensals, opportunistic pathogens or primary pathogens associated with respiratory disease, otitis, mastitis, arthritis, reproductive disease, meningitis and conjunctivitis in cattle [5,11,13]. Some *Mycoplasma* species are highly contagious, spread rapidly within a herd, and respond poorly to antibiotic therapy, which often determines the need of segregating or culling affected animals[13]. Hence, a rapid and accurate diagnosis is of outmost importance both for the control and for prevention of disease outbreaks. Early studies using biochemical methods detected the presence of *M. bovis* associated with mastitis in dairy cattle in Argentina[3]. In the last few years, *Mycoplasma* infections in cattle have received increasing attention, being the organisms identified either by biochemical and molecular methods[12,14,15] or by immunohistochemistry[10].

Several conventional PCR methods have been developed in different countries to identify *Mycoplasma* species either from isolates grown through classical culture or directly from milk samples[13]. However, so far only few *Mycoplasma* species have been isolated from dairy cattle in Argentina and there is scarce information about their frequency and the epidemiological and clinical features associated with their presence in local dairy herds. Hence, there is a need to generate methods for rapid identification at the species level to be used as a diagnostic tool both during outbreaks and epidemiological surveys. We have recently reported the isolation of *M. leachii* associated with arthritis in dairy calves and the development of a multiplex PCR for detecting both this organism and *M. californicum*[12]. Since other species have been reported to be associated with diseases in dairy cattle in Argentina[10,14,15], the objectives of this study were to develop a multiplex PCR for identifying other *Mycoplasma* species (*M. bovis* and *M. canadense*) and to describe the frequency of *Mycoplasma* species isolated from clinical samples submitted to a diagnostic laboratory.

A total of 1548 samples were submitted to the Microbiology Laboratory of the Rafaela Experiment Station of INTA from January 2009 to July 2016 by veterinarians who suspected the presence of *Mycoplasma* as etiologic agent of diseases in dairy cattle. One hundred and seven of these samples were from bulk tank milk, 1412 from composite milk from individual cows and 29 from various organs and biological fluids of dairy calves. The samples came from dairy farms located in the central dairy area of Argentina (Santa Fe and Córdoba provinces) and in the Mar y Sierras dairy area (Buenos Aires province). In a first stage, biological specimens were processed by the classical methodology using modified Hayflick medium at 37 °C in a 10% CO_2 atmosphere incubated for 7–10 days and Mycoplasmas were presumptively identified on the basis of their colony morphology under a microscope at 15–25× magnification as previously described[12]. DNA from bacterial growth in culture medium from colonies suspected to be *Mycoplasma* spp. was extracted using the procedure previously described[12] and subjected to a PCR test to confirm that isolates belonged to this genus[2]. For further identification of *M. leachii* and *M. californicum* a multiplex PCR to amplify specific DNA fragments from the conserved spacer region between the 16S and 23S ribosomal RNA gene was carried out as previously described[12].

For designing a multiplex PCR for amplifying specific DNA fragments of *M. bovis* and *M. canadense* two steps were followed. First, the conserved spacer regions between the 16S and 23S ribosomal RNA gene[1] of *M. bovis* ATCC 25025 and of six unidentified isolates previously characterized as belonging to genus *Mycoplasma* by a genus-specific PCR[2] were amplified and sequenced (ABI3130xl, Applied Biosystems). The sequences obtained were aligned against the NCBI database using BLASTN and then compared with the reported hits in GenBank using the ClustalX multiple sequence alignment program. The 16S-23S rRNA gene sequence obtained from the unidentified strain showed 99% identity with *M. canadense* strain 275C (DQ847417) while the other was confirmed as *M. bovis*. The analysis of the 16S-23S rRNA region allowed the design of species-specific primers, but with very similar amplicon size. Then, specific primers only for *M. canadense* were designed using FastPCR (https://primerdigital.com/tools/pcr.html) for the 16S-23S rRNA region. A multiplex-reaction was set up using those specific primers for *M. canadense* while primers for *M. bovis* were those previously described to amplify a conserved segment of the *uvrC* gene suitable for specific diagnosis[17]. Primers for *M. bovis* were UVRF (5′-TTACGCAAGAGAATGCTTCA-3′) and UVRR (5′-TAGGAAAGCACCCTATTGAT-3′) and for *M. canadense* were CANDF (5′-GCGGAACATTAGTTAGTTGGTA-3′) and CANDR (5′-CGTTAGCTGCGTCAGTGAATT-3′). Expected DNA amplification fragments were 1626 bp for *M. bovis* and 623 bp for *M. canadense*, respectively. The amplification program was: 94 °C 2 min, 30 cycles of 94 °C 30 s, 54 °C 30 s, 72 °C 60 s, 72 °C 5 min. Primers designed for amplifying specific fragments of

Table 1 Frequency and origin of *Mycoplasma* isolated from biological samples obtained from dairy cattle.

Mycoplasma species	Number and percentage of total isolates	Origin (number of isolates)
M. bovis	3 (7.9%)	Bulk tank milk (2), arthritis (1)
M. californicum	6 (15.8%)	Intramammary infection (4), bulk tank milk (2)
M. canadense	1 (2.6%)	Intramammary infection (1)
M. leachii	28 (73.7%)	Intramammary infection (21), bulk tank milk (6), mandibular abscess (1)
Total	38 (100%)	

the 16S-23S rRNA region for differentiation of *M. canadense* as well as those previously described[12] for *M. californicum* and *M. leachii* were tested for specificity using DNA from the other *Mycoplasma* species included in this study. The individual 25 μl reaction mixture consisted of: 2.5 μl of 10× enzyme buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.03 μM of each primer, 1.25 U of *Taq* DNA polymerase (Productos Bio-Lógicos®) and 50 ng of genomic DNA. Cycling consisted of an initial denaturation step at 94 °C for 2 min, and then 30 cycles of the following sequence: 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. In all reactions a negative control using the same previously described mixture, but replacing the DNA by sterile distilled water, was used. The DNA products obtained were visualized in 1.5% agarose gels. The gels were prepared in 1× TAE buffer, adding GelRed TM 10 000× to a final concentration of 1×, the electrophoretic runs were performed at 90 V. A 100 bp ladder step marker was used. DNA fragments resolved in the gel were visualized in a UV light transilluminator (255 nm).

Of the total samples processed, 38 isolates were phenotypically identified as *Mycoplasma* spp. This preliminary identification was confirmed by the genus-specific PCR technique. Identification of the isolates at species level was achieved using both multiplex PCR described. Table 1 summarizes the frequency of isolated *Mycoplasma* species and their origin.

Since convenience samples were used, the results of this study are not indicative of the prevalence of the different isolated species. The low number of isolates in relation to the total of processed samples indicates that the presence of this genus detected by classical culture is not a frequent finding. No systematic studies have been performed in Argentina to determine prevalence of the different *Mycoplasma* species associated with defined syndromes in dairy cattle; rather, disease outbreaks and clinical cases have been reported so far. Three isolates (7.9%) were identified as *M. bovis*. Within the *Mycoplasma* genus, *M. bovis* is currently considered the most frequently isolated species associated with disease in cattle worldwide[11]. Since the first report causing mastitis in a dairy herd, *M. bovis* has been isolated from cases of respiratory disease, mastitis and arthritis in cattle, being considered an emergent pathogen in several countries[5,11]. In Argentina its presence was first reported in a mastitis outbreak in a dairy farm located in Buenos Aires province where 11.8% of cows presented clinical mastitis that in various cases affected the four mammary quarters, caused a marked decrease in milk production and did not respond to antibiotic therapy. The organism was isolated from composite milk samples from cows with clinical and subclinical mastitis, bulk tank milk and a preputial swab from a bull by classical culture and characterized as *M. bovis* by biochemical tests and indirect immunofluorescence of the isolated colonies[3]. More recently, *M. bovis* was detected in milk samples both from cows with clinical and subclinical mastitis and bulk tank milk from dairy herds from Buenos Aires and Córdoba provinces and characterized by amplifying a fragment of the *uvrC* gene[15]; however, no further information was provided about the cases from which the processed samples came. In addition, *M. bovis* was identified by immunohistochemistry in cases of bronchopneumonia unresponsive to antibiotic treatment both in beef and dairy calves from Córdoba and Santa Fe provinces[10]. These results, together with those obtained in the present study confirm the importance of *M. bovis* as a causative agent of mastitis, bronchopneumonia and arthritis as has been pointed out in different countries[5,11] and indicate the need to include diagnostic methodology in routine laboratories to identify this pathogen.

M. canadense was isolated from a single case of intramammary (IMI) infection from a dairy herd in Buenos Aires province. Diseases and clinical cases associated with this organism are not as well characterized as those associated with *M. bovis*. This species has been reported to cause not only IMI[5], but also to be associated with granulopapular vulvovaginitis in dairy cattle in single or mixed infections with *Mycoplasma bovigenitalium*[8] and with arthritis in a calf[9]. In Argentina, this species was isolated from two bulk tank milk samples from a dairy farm located in Buenos Aires province[15].

M. californicum is considered to be the second most frequently isolated *Mycoplasma* species from bovine mastitis cases in USA after *M. bovis*[5], sharing with the latter similar characteristics regarding both the clinical mastitis features and the importance of the presence of asymptomatic carriers in the dissemination of the organism among cows and dairy herds[6]. In the present study, this organism was isolated from 4 cows during an outbreak of mastitis refractory to antibiotic therapy, as well as from two bulk tank milk samples from the same dairy farm located in Córdoba province. In Argentina, the isolation of *M. californicum* was reported from bulk tank milk samples[15] and clinical mastitis samples from the same dairy herd[14] located in Buenos Aires province.

Twenty eight isolates (73.7%) were characterized as *M. leachii*. These isolates belonged to a single dairy farm where cases of polyarthritis in calves were previously detected[12].

In this farm, milk samples were taken from clinical mastitis cases and also from individual cows (composite milk samples) as part of a control program based on the detection of asymptomatic carriers. In addition, bulk tank milk samples were taken for monitoring the presence of the organism in the lactating dairy herd as the control program progressed. *M. leachii*, previously referred to as *Mycoplasma* sp. bovine Group 7[16], was early recognized as a cause of polyarthritis, mastitis and abortion in dairy cattle in Australia[7] and recent studies characterized clinical signs and histopathological lesions induced by experimental IMI with this pathogen[4]. The present study, as well as our previous report[12] confirms the ability of this organism to cause diverse clinical syndromes.

In conclusion, the multiplex PCR technique for detecting *M. bovis* and *M. canadense* described in this study, along with the one previously described for detecting *M. californicum* and *M. leachii*, provide a useful diagnostic tool for characterizing some of the *Mycoplasma* species that cause disease in cattle in Argentina. The results of this study confirm the presence of several species of *Mycoplasma* associated with different disease syndromes in dairy cattle. The techniques used in the present study can be useful to broaden knowledge about *Mycoplasma* infections in cattle, since the search for these organisms is not usually included in routine diagnoses.

Conflict of interest

The authors declare that they have no conflicts of interest.

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