

## *Mycoplasma* isolation in milk samples from dairy herds in Chile

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**ABSTRACT.** *Mycoplasma* bovine mastitis is a highly contagious disease, usually associated with clinical cases refractory to antibiotic treatment. The aim of this study was the isolation of *Mycoplasma* species in cattle milk samples from dairy herds in Chile. Bulk tank milk samples selected by convenience from 91 Holstein Friesian dairy herds located in Los Rios (66) and Los Lagos (25), the two most important dairy Regions in the country, were collected. Additionally, 100 individual milk samples from cows with a high incidence of clinical mastitis, refractory to antibiotic therapy, and negative bacteriological results for traditional mastitis pathogens, all from the Biobío Region and received in our diagnostic laboratory, were included. All samples were cultured for 10 days on PPLO medium. The differentiation of suspect colonies between genus *Mycoplasma* and *Acholeplasma* was performed by the digitonin test and a specific PCR. The species identification was performed by a *M. bovis* specific PCR and 16S rRNA sequencing. *Mycoplasma* was isolated from 3 (3.3%) bulk tank milk samples and 2 (2%) individual cow milk samples. All colonies were identified as *Mycoplasma* by the digitonin test and by a specific PCR. At species level, one strain isolated from a bulk tank milk sample was identified as *M. bovis*. The remaining two strains isolated from bulk tank milk samples were identified as *M. bovigenitalium*, while the two strains isolated from milk of individual cows were identified as *M. alkalescens*. These results show that not only *M. bovis* is present in Chilean dairy herds, but also other pathogenic species not previously described in Chile such as *M. bovigenitalium* and *M. alkalescens*, which pose a potential risk for dairy herds in southern Chile.

*Key words:* bovine mastitis, *Mycoplasma* mastitis, dairy herds.

### INTRODUCTION

Bovine intramammary infection due to *Mycoplasma* is an emergent problem in the dairy industry of many countries. In recent years, an increase in *Mycoplasma* mastitis prevalence has been observed especially associated with large dairy herds (Fox *et al* 2003, Lysnyansky *et al* 2016, Nicholas *et al* 2016, Timonen *et al* 2017, Gille 2018, Abd El Tawad 2019). This type of infections cause major economic losses because are highly contagious, cannot be detected with conventional culture media, do not respond to antibiotic treatments, can affect multiple quarters, produce a large decrease in milk yield, and infected animals usually must be segregated or culled (Nicholas *et al* 2016). Common species of *Mycoplasma* isolated from intramammary infections in cows are *M. bovis*, *M. californicum*, *M. bovigenitalium*, *M. alkalescens* and *M. canadense*. However, *M. bovis* is the most frequent species and the one that produces the most severe clinical cases (Fox 2012). In cattle, *M. bovis* has also been associated with pneumonia, arthritis, otitis and reproductive disorders (Nicholas and Ayling 2003, Maunsell *et al* 2011).

*Mycoplasmas* are one of the smallest known microorganisms, they lack a cell wall and require special

media for their *in-vitro* growth (Razin *et al* 1998). The routine bacteriological diagnosis procedure for *Mycoplasma* mastitis is the cultivation of milk samples on special media which are incubated for 7-10 days at 37 °C with 10% CO<sub>2</sub> (Hogan *et al* 1999). The morphological diagnosis is based on recognition of the typical “fried eggs” appearance of the *Mycoplasma* colonies, and its subsequent identification can be done by serological methods, PCR techniques, 16S rRNA gene sequencing, MALDI-TOF and whole genome sequencing (WGS) (Boonyayatra *et al* 2012, Nicholas *et al* 2016, Parker *et al* 2018).

Although the culture of milk samples has a low sensitivity, surveillance using bulk tank milk samples culture for *Mycoplasma* detection is essential in a *Mycoplasma* mastitis control program (Fox *et al* 2003, Wilson *et al* 2009, Fox 2012). The *Mycoplasma* mastitis prevalence, based on bulk tank milk culture, varies between countries, e.g. Belgium 1.5% (Passchyn *et al* 2012), Israel 2.7% (Lysnyansky *et al* 2016), USA 3.2% (APHIS-USDA. 2008), Japan 3.8 % (Murai and Higuchi 2019) and Grece 5.4% (Filiouis *et al* 2007). Sporadic outbreaks have been recently described in some countries but they may be underreported (Nicholas *et al* 2016).

In Chile, *M. bovis* had been previously reported in bulk tank milk samples by Sickles *et al* (2000) and later Bustos and Muñoz (2011) described *Mycoplasma* spp. in bulk tank milk samples in dairy herds of the Biobio Region. Despite clinical cases of bovine mastitis that are refractory to treatment and culture negative are common in Chilean dairy herds, no more data are available about the presence of *Mycoplasma* in bulk tank milk and cow milk samples. The aim of this study was the isolation of *Mycoplasma* species in cattle milk samples from dairy herds in Chile.

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## MATERIAL AND METHODS

### MILK SAMPLES COLLECTION

Ninety-one Holstein Friesian dairy herds from Los Rios (66) and Los Lagos (25), the two most important dairy Regions in the country, were selected by convenience. The herds had between 50 and 400 lactating cows and were providers of the “Sociedad Procesadora de Leche del Sur S.A” (PROLESUR). Most of them were managed under grazing-based systems with concentrate and silage as winter supplementation. Individual milk samples were collected from each bulk milk tank by trained personnel from the PROLESUR company in accordance with the recommendations of the National Mastitis Council, USA (Hogan *et al* 1999). All the samples were kept and transported at 4 °C to the laboratory within 8 h of collection for bacteriological examination. Additionally, 100 individual milk samples from cows with >500,000 cells/mL and negative bacteriological results for traditional mastitis pathogens were included. All these milk samples were received in our diagnostic laboratory and came from a dairy herd located in the Biobío Region, with a high incidence of clinical mastitis refractory to antibiotic therapy.

### BACTERIOLOGICAL ANALYSIS

Immediately after arrival at the laboratory, 100 µL of each milk sample were plated onto the surface of PPLO Medium (Difco), supplemented with yeast extract, horse serum, salmon DNA, thallium acetate and penicillin and incubated at 37 °C with 10% CO<sub>2</sub> (Hogan *et al* 1999). The plates were read at 3, 7, 10 and 12 days of incubation using a stereoscopic microscope (40x), observing all the streaks made on the agar to detect *Mycoplasma* colonies. At the end of the incubation period, the plates with no growth were discarded and those growing small colonies with a “fried egg” appearance were considered suspicious of *Mycoplasma*. Then, the surrounding agar of the suspicious colonies was cut out and subcultured into PPLO broth supplemented as mentioned above, and incubated for 3 days at 37 °C with 10% CO<sub>2</sub>, to obtain a pure culture. *M. bovis* ATCC 25025 strain was used as a positive control for all the tests.

For DNA extraction, 5 mL of a pure culture was centrifuged at 12,000 g for 30 seconds, the supernatant discarded, and the pellet resuspended in 150 µL of PBS, and processed with the commercial kit “AxyPrep Bacterial Genomic DNA Mini-Preparation Kit” (Axygen, Inc.) following the manufacturer instructions.

### GENUS IDENTIFICATION

For the Genus differentiation between *Mycoplasma* and *Acholeplasma*, a common environmental organism, the digitonin disk test and a specific PCR test were used

(Boonyayatra *et al* 2012). The digitonin disk test was performed using a sterile cotton swab moistened with pure culture broth of the suspicious colony, which was inoculated onto the entire surface of a PPLO agar plate. Afterwards, a digitonin disk (Udder Health System, Inc) was placed in the centre of the plate and incubated for 4 days at 37 °C with 10% CO<sub>2</sub>. The presence of a growth inhibition zone surrounding the digitonin disk was visually checked. A clear inhibition zone >5 mm from the edge of the disk was considered as a positive test for the genus *Mycoplasma*. On the contrary, when the growth inhibition zone was <3 mm it was considered as a negative test indicating that the strain belongs to the genus *Acholeplasma*. In addition, a specific PCR test for genus differentiation was performed using the set of primers F2 5'-GTG(C/G)GG(A/C)TGGATCACCTCCT-3' and R2 5'-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3' which targets the 16S-23S rRNA intergenic spacer region of *Mycoplasma* and R34 5'-CCACTGTGTGCCCTTTGTTTCCT-3' of the 16S-23S rRNA intergenic spacer region of *Acholeplasma*. (Boonyayatra *et al* 2012). The amplification program was an initial denaturation cycle of 5 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 2 minutes at 55 °C and 2 minutes at 72 °C, with a final extension of 5 minutes at 72 °C. Amplification products were separated by 2% agarose gel electrophoresis stained with ethidium bromide. The presence of two bands was considered a positive result for *Acholeplasma*, while *Mycoplasma* produces only one band.

### SPECIES IDENTIFICATION

Once the suspect strains were confirmed as *Mycoplasma*, a specific PCR was performed for the identification of *M. bovis*. The primers mb-mp 1F 5'-TATTGGATCAACTGCTGGAT-3' and mb-mp 1R 5'-AGATGCTCCACTTATCTTAG-3' were used for the amplification of the mb-mp 81 gene, as described by Foddai *et al* (2005). The thermocycler was set with an initial denaturation cycle of 5 minutes at 94 °C, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 54 °C and 1 minute at 72 °C, with a final extension cycle of 10 minutes at 72 °C. The amplification product was separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The presence of a band of approximately 447 bp was considered as *M. bovis*. Additionally, all strains were identified by sequencing the 16S rRNA gene, according to the protocol described by Botes *et al* (2005). PCR products of approximately 1048 bp in size were purified using the commercial kit Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Once the PCR products were purified, they were sequenced bidirectionally in MACROGEN (Korea). Consensus sequences were compared to sequences deposited at GenBank using the NCBI's nucleotide-nucleotide BLAST program. An identity above 98.6% was considered as the same *Mycoplasma* species (Kim *et al* 2014).

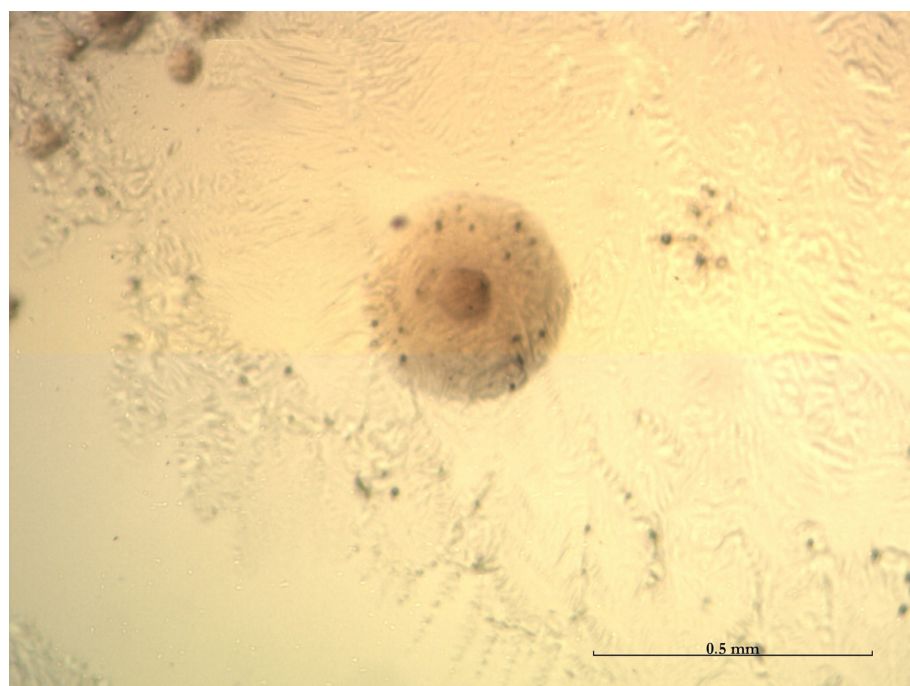
## RESULTS AND DISCUSSION

*Mycoplasma* was isolated in 3 out of 91 (3.3%) bulk tank milk samples. Typical colonies were 300-400 µm in diameter, with a dense central nucleus, surrounded by a lighter peripheral area of growth, which gives it the characteristic “fried egg” appearance (figure 1). Additionally, *Mycoplasma* was also isolated in 2 out of 100 (2%) individual milk samples from cows with subclinical mastitis. All colonies were identified as belonging to the *Mycoplasma* genus by both the digitonin and PCR tests. These results show that *Mycoplasma* is present in dairy herds of southern Chile, however, it must be considered that its presence may be underestimated due to the low sensitivity of a single bulk tank culture. Despite this disadvantage, the quality of the *Mycoplasma* bulk milk tank culture can be improved by

performing three consecutive cultures separated by 3-4 days (Gonzalez and Wilson 2002).

The three isolated strains from bulk tank milk samples were named J14, J35 and J71. Regarding the species identification, only strain J35 was identified as *M. bovis* by the specific PCR, which produced a band of approximately 447 bp (data not shown), and by partial sequencing of the 16S rRNA gene. Strains J14 and J71 were identified at the species level as *M. bovisgenitalium* by partial sequencing of the 16S rRNA gene. The two strains isolated from cases of subclinical mastitis were named V49 and V61, both identified as *M. alkalescens* by partial sequencing of the 16S rRNA gene (table 1).

In Chile, *M. bovis* had been isolated in 5 out of 71 (7%) bulk milk tank samples of herds from Los Ríos and Los Lagos Regions, from different dairy farms (Sickles *et al*



**Figure 1.** *Mycoplasma* colony on PPLO agar plate, with typical “egg fried” appearance (400x).

**Table 1.** Identification of the isolated mycoplasma strains.

Strain name	Source	Digitonin	PCR <i>Mycoplasma</i> spp.	PCR <i>M. bovis</i>	16s rRNA gene sequencing		
					Aligned strain	Access	Similarity
J14	BTM*	+	+	-	<i>M. bovisgenitalium</i> NBRC 14862	AB680692.1	99%
J35	BTM*	+	+	+	<i>M. bovis</i> PG 45	NR102850.1	99%
J71	BTM*	+	+	-	<i>M. bovisgenitalium</i> NBRC 14862	AB680692.1	99%
V49	SCM**	+	+	-	<i>M. alkalescens</i> PG51	NR025984.1	100%
V61	SCM**	+	+	-	<i>M. alkalescens</i> PG51	NR025984.1	100%

\*Bulk tank milk.

\*\*Subclinical mastitis.

2000). The percentage of *Mycoplasma* positive milk tanks was higher in this study, but the results are not comparable because in our study we used only samples from suppliers of PROLESUR company. Another Chilean study reports that *Mycoplasma* was isolated from 4 out of 11 (36%) bulk tank milk samples from the Biobío Region, but the strains isolated were not identified at species level (Bustos and Muñoz 2011). Although the percentage of *Mycoplasma*-positive bulk tanks found in our study was similar to those reported in other countries (Fox 2012), further studies are needed to find out the real prevalence of *Mycoplasma* in Chile.

We found only one bulk tank milk positive for *M. bovis*, which indicates that there is, at least, one cow with *Mycoplasma* mastitis in the herd, so it would be advisable to identify all infected animals and segregate or remove them from the herd (Fox 2012, Nicholas *et al* 2016). *M. bovis* causes more severe cases of intramammary infections due to *Mycoplasma*, and has been associated with respiratory infections, otitis, septic arthritis, and reproductive infections in cattle (Nicholas and Ayling 2003, Maunsell *et al* 2011). Intramammary infection due to *Mycoplasma bovis* is feared by dairy farmers and veterinarians because it is highly contagious, hard to control and there are still no vaccines available, as for other udder pathogens (Mella *et al* 2017).

In addition, this study describes for the first time in Chile the isolation of *M. bovisgenitalium* and *M. alkalescens*. *M. bovisgenitalium* has been documented as a cause of diseases in dairy herds, mainly associated to genital tract infections (Lysnyansky *et al* 2009) and cases of clinical mastitis (Baumgartner *et al* 2006, Lysnyansky *et al* 2016). It is one of the three most isolated *Mycoplasma* species from bulk milk tanks, with an isolation frequency that varies between 1% and 25% (Fox 2012). Occasionally, *M. bovisgenitalium* and *M. alkalescens* can be isolated from milk samples, but not always their presence is associated with disease (Lysnyansky *et al* 2016). However, in our report *M. alkalescens* was isolated from cows with subclinical mastitis that had negative cultures results to other common mastitis pathogens, so it is likely to be the causative agent of intramammary infection in these animals.

In conclusion, *Mycoplasma* mastitis is a potential risk for dairy herds in southern Chile since, although in low percentages, the agents are present in the herd environment. It is important to note that not only *M. bovis* is present in these dairy herds, but also other pathogenic species not previously described in Chile such as *M. bovisgenitalium* and *M. alkalescens*. Consequently, diagnostic laboratories should attempt to isolate *Mycoplasma* spp. when testing milk samples from cows with clinical mastitis that do not respond to antibiotic treatment.

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