



BRIEF REPORT

Detection of *Mycoplasma canadense* and *Mycoplasma californicum* in dairy cattle from Argentina

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Abstract

Different species of *Mycoplasma* can affect bovine cattle, causing several diseases. PCR sequencing and further analysis of the 16S-23S rRNA ITS region have shown a significant interspecies variability among *Mollicutes*. Sixteen suspected isolates of *Mycoplasma* spp. obtained from milk samples from dairy herds were amplified (16S-23S rRNA ITS region). Fourteen out of those 16 suspected *Mycoplasma* spp. isolates were PCR-positive. To confirm the identity of *Mycoplasma bovis*, these 14 isolates were tested by another species-specific PCR. Seven of the isolates rendered a positive result. The products of 16S-23S rRNA ITS PCR from one isolate that was identified as *M. bovis* and from two other isolates, identified as non- *M. bovis* were randomly selected, sequenced and analyzed. The three sequences (A, B and C) showed 100% similarity with *M. bovis*, *Mycoplasma canadense* and *Mycoplasma californicum* respectively.

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PALABRAS CLAVE

Ganado lechero;
Mycoplasma bovis;
Mycoplasma canadense;
Mycoplasma californicum;
ITS;
16S-23S ARNr

Detección de *Mycoplasma canadense* y *Mycoplasma californicum* en ganado lechero de Argentina

Resumen

Diferentes especies del género *Mycoplasma* pueden afectar al ganado bovino y causar varias enfermedades. La técnica de PCR, secuenciación y posterior análisis de la región ITS 16S-23S ARNr ha mostrado que existe una importante variabilidad interespecies entre *Mollicutes*. Se realizó la amplificación (región ITS 16S-23S ARNr) de 16 aislamientos sospechosos de corresponder a alguna especie de *Mycoplasma*, que habían sido obtenidos de muestras de leche provenientes de rodeos lecheros. Catorce de esos aislamientos fue-

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ron PCR positivos. Para confirmar la identidad de *Mycoplasma bovis*, dichos aislamientos fueron evaluados por otra PCR especie-específica. Siete aislamientos dieron un resultado positivo. Los productos de la PCR de la ITS 16S-23S ARNr de un aislamiento identificado como *M. bovis* y de otros dos aislamientos identificados como no-*M. bovis* fueron seleccionados al azar, secuenciados y analizados. Las tres secuencias (A, B y C) mostraron 100 % de similitud con cepas de *M. bovis*, *Mycoplasma canadense* y *Mycoplasma californicum*, respectivamente.

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Different species of *Mycoplasma* can affect bovine cattle, causing several diseases. Mycoplasmas can cause clinical, subclinical or chronic intramammary infection affecting cattle of all ages and at any stage of lactation³. In Argentina, *Mycoplasma bovis* was firstly reported in the year 2000² in a bovine mastitis outbreak in Buenos Aires province. Since then, *Mycoplasma* spp. has been frequently isolated but there are no literature reports about the identification of other *Mycoplasma* species affecting bovine cattle.

As in bacteria, in mycoplasmas the *rRNA* genes (16S-23S-5S) are separated by internal transcriber spacer (ITS) regions¹⁰. Sequencing and analysis of the 16S-23S rRNA ITS region have shown a significant interspecies variability among *Mollicutes*¹³. In fact, this region has been proposed as a complementary genetic marker for species identification of the genera *Mycoplasma*¹⁴.

In our laboratory, we routinely use the amplification of a fragment of the 16S-23S ITS region as a screening PCR test for *Mycoplasma* spp. identification from isolates or clinical samples from dairy cattle, in which *Mycoplasma* infection is suspected. Due to the novelty of our findings and the lack of information about the *Mycoplasma* species affecting dairy cattle in our country, the objective of this study is to report the detection of *Mycoplasma bovis*, *Mycoplasma canadense* and *Mycoplasma californicum* in dairy cattle from Argentina, by sequencing the 16S-23S rRNA ITS region of the isolates.

This work was performed at the Laboratory of Animal Pathology of the Faculty of Agronomy and Veterinary Sciences (UNRC, Río Cuarto, Córdoba, Argentina), according to the international guidelines of the Council for International Organizations of Medical Sciences (CIOMS).

Culture and DNA extraction. Sixteen suspected isolates of *Mycoplasma* spp. obtained from milk samples from dairy herds were analyzed. Milk samples were taken from mammary quarters of cows with and without clinical mastitis and from bulk tank milk from herds from Córdoba and Buenos Aires provinces. These samples were cultured in Modified Hayflick's medium plates at least 7 days at 36 +/- 1 °C with 10% CO₂. *Mycoplasma* spp. suspected colonies were identified by daily examination of plates under a stereomicroscope. These colonies were picked up and inoculated into Modified Hayflick broth medium and incubated for 48 hs at 36 +/- 1 °C with 10% CO₂. One ml of each culture was centrifuged at 8,000 × g for 10 min, pellets were washed twice with PBS solution and suspended in 150 µl of sterile purified water. DNA was extracted by boiling (10 min).

PCR and sequencing. DNA was subjected to PCR for 16S-23S rRNA ITS fragment amplification to identify *Mycoplasma* using the primers and PCR conditions previously described^{4,6,7}.

Fourteen out of 16 suspected *Mycoplasma* spp. colonies were PCR- positive to 16S-23S rRNA ITS amplification reaction. The size of the amplicons varied approximately from 350 bp to 500 bp. To identify *M. bovis*, these 14 colonies were tested by another PCR, amplifying a fragment of the *uvrC* gene using the primers and conditions described by Subramanian *et al.* and Thomas *et al.* respectively^{11,12}. Seven of the colonies rendered a positive result.

PCR products from 16S-23S rRNA ITS amplification from one of the colonies identified as *M. bovis* (isolate A) and two other colonies identified as *Mycoplasma non-M. bovis* (isolates B and C) were randomly selected for further analysis. These three amplicons were purified (Puriprep-GP Kit, InbioHighway, Tandil, Argentina), quantified, and sequenced (ABI 3130xl; Applied Biosystems, Foster City, California) with the primers described by Harasawa *et al.*^{6,7}. The sequences were visualized using the BioEdit software⁵ and were aligned against the database using nucleotide BLAST¹. In order to eliminate the flanking regions (corresponding to partial sequences of the 16S rRNA and 23SrRNA genes) the sequences were aligned using ClustalW⁹.

The 16S-23S rRNA ITS sequence obtained from isolate A showed 100% similarity with the same region of *Mycoplasma bovis* strains PG45 (CP002188.1), 70-213 (AY779747.1), Hubei-1 (CP002058.1), HB0801 (CP002058.1), HEK-FDA (JN644755.1), Madison (AY780798.1), and ATCC 25025 (AY765211.1). These results were in accordance with the identification of this isolate as belonging to *M. bovis* by PCR amplification of *uvrC*.

The 16S-23S rRNA ITS sequence obtained from isolate B showed 100% similarity with the same region of *Mycoplasma canadense* strains QMP-SRI-0053 (KC759701.1), QMP-SRI-0054 (KC771072.1), QMP-SRI-0052 (KC485347.1), 275C (DQ847417.1), ATCC 29418 (AY800341.1), and 466 (EU925158.1; DQ847418.1). The sequence alignment from strain C to *Mycoplasma californicum* strains ST6 (DQ847428.1) and ATCC 33461 (AY736031.1) showed 100% similarity in both cases.

The present study confirms the presence of this pathogen in milk samples by sequencing the 16S-23S rRNA ITS region and also by the amplification of a species-specific gene *uvrC*¹²; however, this case corresponded to an isolate from a cow with clinical mastitis from Córdoba province (data not shown).

It is worth noting that in Argentina, the presence of *M. canadense* and *M. californicum* has never been reported before. *M. canadense* and *M. californicum* together with *M. bovis*, *M. arginini*, *M. bovirhinalis*, *M. bovirhinalis* and *M. alkalescens* are the major *Mycoplasma* species

causing mycoplasmal mastitis around the world. In Latin America the first report of a mastitis outbreak associated to *M. canadense* and *M. californicum* was described in Mexico⁸; however, there is lack of information about its presence in other Latin American countries. In our case, strains B and C had been isolated from bulk tank milk from two different herds from Buenos Aires province (data not shown).

Volokhov *et al.*¹² demonstrated that the ITS is a suitable and valuable marker for species identification among *Mollicutes*, since it showed a high percentage of interspecies diversity, low intraspecies variability and conserved flanking regions. Due to the labor-intensive and time-consuming isolation and biochemical identification of *Mycoplasma* species, their identification by amplification of the 16S-23S rRNA ITS region and further sequencing represents an easier and faster approach.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990;215: 03-10.
- Cerdá R, Xavier J, Sansalone P, de la Sota R, Rosenbush R. Isolation of *Mycoplasma bovis* during an outbreak of bovine mastitis at a dairy farm in the province of Buenos Aires. 1st report in the Republic of Argentina. *Rev Latinoam Microbiol.* 2000;42:7-11.
- González RN, Wilson DJ. Mycoplasmal mastitis in dairy herds. *Vet Clin North Am Food Anim Pract.* 2003;19:199-221.
- Gopalkrishna V, Verma H, Kumbhar NS, Tomar RS, Patil PR. Detection of *Mycoplasma* species in cell culture by PCR and RFLP based method: effect of BM-cyclin to cure infections. *Indian J Med Microbiol.* 2007;25:364-8.
- Hall, T. 2007. BioEdit: Biological sequence alignment editor for Win95/98/NT/2K/XP [online]. Website last modified on June 27, 2007 [accessed 13 Sep 2013]. Available from: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>
- Harasawa R, Mizusawa H, Nozawa K, Nakagawa T, Asada K, Kato I. Detection and tentative identification of dominant *Mycoplasma* species in cell cultures by restriction analysis of the 16S-23S rRNA intergenic spacer regions. *Res Microbiol.* 1993;144:489-93.
- Harasawa, R. Nested PCR. Application to the detection of mycoplasmas. In: Razin S, Tully JG, editors. *Molecular and diagnostic procedures in mycoplasmaology*. Vol. 2. London, Academic Press; 1995. p. A4.
- Infante-Martinez F, Aguado J, Eduard-Jasper D. Mastitis outbreak due to *Mycoplasma californicum* and *Mycoplasma canadense* in a commercial dairy herd in the state of Jalisco, México. *Rev Latinoam Microbiol.* 1999;41:117-20.
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. ClustalW and ClustalX version 2. *Bioinformatics.* 2007;23:2947-8.
- Razin S. Molecular biology and genetics of mycoplasmas (*Mollicutes*). *Microbiol Rev.* 1985;49:419-55.
- Subramaniam S, Bergonier D, Poumarat F, Capaul S, Schlatter Y, Nicolet J, Frey J. Species identification of *Mycoplasma bovis* and *Mycoplasma agalactiae* based on the *uvrC* genes by PCR. *Mol Cell Probes.* 1998;12:161-9.
- Thomas A, Dizier I, Linden A, Mainil J, Frey J, VileiEM. Conservation of the *uvrC* gene sequence in *Mycoplasma bovis* and its use in routine PCR diagnosis. *Vet J.* 2004;168:100-2.
- Volokhov DV, George J, Liu SX, Ikonomi P, Anderson C, Chizhikov V. Sequencing of the intergenic 16S-23S rRNA spacer (ITS) region of *Mollicutes* species and their identification using microarray-based assay and DNA sequencing. *Appl Microbiol Biotechnol.* 2006;71:680-98.
- Volokhov DV, Hong H, George J, Anderson C, Davidson M, Chizhikov V. The 16S-23S rRNA intergenic transcriber region (ITS), *rpoB* and *gyrB* genes as complementary genetic markers to the 16 rRNA gene for phylogenetic analysis and species identification of members of the genera *Mycoplasma*, *Ureoplasma*, *Acholeplasma*, *Entomoplasma* and *Mesoplasma*. *Proc of 18th Congress of the International organization of Mycoplasmaology.* 2010. Poster session II. 151, p. 103, ChiancianoTerme, Siena, Italy.