



BRIEF REPORT

## First report of *Mycoplasma leachii* isolation associated with disease in dairy calves in Argentina



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### PALABRAS CLAVE

*Mycoplasma leachii*;  
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Terberos de tambo

**Abstract** There are few reports about the isolation of *Mycoplasma* species associated with cattle disease in Argentina. In this work we describe the detection of *Mycoplasma leachii* associated with disease in dairy calves in Santa Fe Province, Argentina. Samples obtained from a 4 day-old dairy calf suffering from polyarthritis and from two other calves, one with arthritis and the other one with a mandibular abscess, were subjected to microbiological culture. Classical culture and generic PCR confirmed the presence of *Mycoplasma* spp. The spacer region between the 16S and 23S ribosomal RNA gene from the first isolate was amplified and sequenced. The sequence obtained showed 99% identity with *M. leachii*. A PCR was developed to amplify a specific fragment of the 16S-23S ITS region corresponding to *M. leachii*, which allowed to identify the isolates associated with disease in calves.

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### Primer aislamiento de *Mycoplasma leachii* asociado a enfermedad en terneros de tambo en Argentina

**Resumen** Existen pocos informes acerca del aislamiento de especies de *Mycoplasma* asociadas con enfermedades del ganado en Argentina. En esta comunicación se describe el aislamiento de *Mycoplasma leachii* asociado a enfermedad en terneros de tambo en la provincia de Santa Fe, Argentina. Se obtuvieron muestras de un ternero de 4 días de vida con poliartritis, de un ternero con artritis y uno con un absceso mandibular. A partir del cultivo clásico se detectó la presencia de *Mycoplasma*, lo cual fue confirmado por PCR genérica. Se amplificó y secuenció la región ITS 16S-23S a partir del primer aislamiento, mostrando una identidad del 99% con

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*Mycoplasma leachii*. Se desarrolló una PCR para amplificar un fragmento específico de la región ITS 16S-23S correspondiente a *M. leachii*, que permitió identificar los aislamientos asociados con enfermedad en terneros.

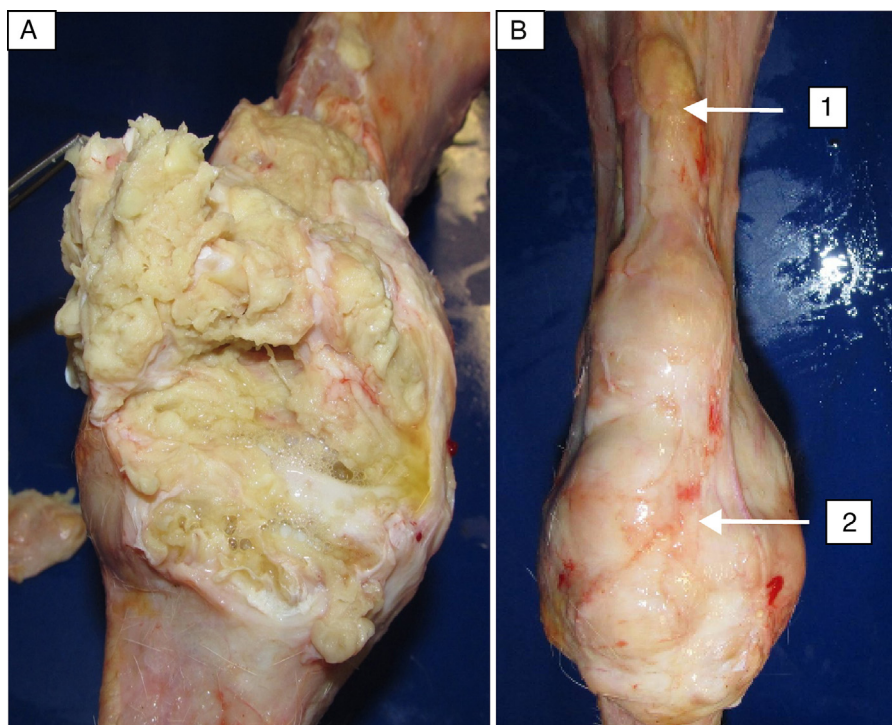
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Mycoplasmas belong to the class *Mollicutes* and are the smallest self-replicating prokaryotes that exist in nature, being parasites of mammals, birds, reptiles, fish, arthropods and plants<sup>3</sup>. These organisms, which are typical inhabitants of the respiratory, urogenital, conjunctival, gastrointestinal and mammary gland mucosal surfaces, have been early recognized as a cause of respiratory disease, otitis, mastitis, arthritis, reproductive disease, meningitis, and conjunctivitis in cattle<sup>3,7</sup>.

There are few reports about the isolation of *Mycoplasma* species associated with disease in cattle in Argentina. The detection of *Mycoplasma dispar* from calf pneumonia cases in Buenos Aires province was early reported<sup>1</sup>. More recently, *Mycoplasma bovis* was isolated from a bovine mastitis outbreak that did not respond to antibiotic therapy<sup>4</sup> and *Mycoplasma canadense* and *Mycoplasma californicum* were isolated from milk samples obtained from cows with mastitis<sup>12</sup>. The aim of this report was to describe the classical microbiological and molecular detection of *Mycoplasma leachii* associated with disease in dairy calves in Santa Fe Province, Argentina.

Case 1: a 4-day old Holstein calf belonging to a dairy herd from the Central dairy area (Santa Fe province, Argentina) presented a left carpal inflammation that became bilateral with joint enlargement and difficulty to move. In the third week of life the animal was admitted to the Animal Health Hospital at the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral, showing polyarthritis characterized by joint enlargement, high rectal temperature and reluctance to rise. The main clinical features were: joint swelling, intermittent rise in rectal temperature, dyspnea that was more noticeable on exhalation, tachycardia and poor body condition. Synovial fluid was aseptically obtained by needle aspiration and submitted for microbiological culture. Upon admission to the Hospital antibiotic and anti-inflammatory therapy with gentamicin and flunixin meglumine, respectively, were administered. As disease progressed, since lack of response was observed, the antibiotic was replaced by tylosin. The calf died 35 days after the onset of clinical signs and samples from synovial capsule, lymph nodes and lung were aseptically taken at necropsy for microbiological analysis. Case 2: eight months after the first clinical case, two calves of approximately 30 days of age, from the same dairy herd were also clinically ill. Lesions corresponded to carpal arthritis and a mandibular abscess. Samples were obtained by needle aspiration and submitted for microbiological culture. All procedures involving animals were carried out according to the Guide for the Care and Use of Laboratory Animals<sup>11</sup>.

Synovial fluid, abscess content and organs were subjected to direct microscopic observation following Gram and Giemsa staining. Samples were seeded on Columbia agar supplemented with 5% defibrinated bovine blood and incubated aerobically and in 10% CO<sub>2</sub> for 72 h. In addition, samples were seeded on Modified Hayflick's agar and incubated in 10% CO<sub>2</sub> for 7 days. DNA from morphologically typical *Mycoplasma* colonies was extracted by a previously described procedure<sup>14</sup> with slight modifications. Briefly, in a first step, bacterial frozen stocks kept at -76 °C were activated in 2 ml modified Hayflick's broth for 24–48 h at 37 °C in a 10% CO<sub>2</sub> atmosphere. A bacterial pellet was obtained by centrifugation at 14 000 × g for 10 min, washed twice with PBS and resuspended in 40 µl PBS. The suspension was subjected to heating at 100 °C for 10 min, followed by cooling at -20 °C for 10 min and then again centrifuged at 12 000 × g for 2 min. A PCR test was carried out to confirm that isolates belonged to genus *Mycoplasma*<sup>13</sup>. Isolates that yielded a positive result were further tested by another PCR to amplify a fragment of the *uvrC* gene of *M. bovis*<sup>14</sup>. If this specific fragment was not amplified, the spacer region between the 16S and 23S ribosomal RNA gene was amplified<sup>2</sup> and sequenced (ABI3130xl, Applied Biosystems). The sequences obtained were aligned against the NCBI database using BLASTN and compared with those reported in GenBank using the Clustal X multiple sequence alignment program. The 16S-23S rRNA gene sequence obtained from the first isolate recovered from Case 1 showed 99% identity with *M. leachii*. To further identify the rest of the isolates obtained from Cases 1 and 2, primers were designed using FastPCR for amplifying specific sequences of *M. leachii* and *M. californicum* from this conserved region. The latter was included as a control. Primers for *M. leachii* were MycoF (GCCGAAGTGA-GAGGTTGAT) and MycoR (ATAACGCTTGCCACCTATGT) and for *M. californicum* were CalifF (CCACCAAGGCAATGATGTT) and CalifR (TGTCAGTATATGTCCAGTTAGC). Expected DNA amplification fragments were 266 bp for *M. leachii* and 490 bp for *M. californicum*, respectively. Each individual 25 µl reaction mixture consisted of: 2.5 µl of 10× enzyme buffer, 2 mM MgCl<sub>2</sub>, 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. Thermocycling consisted of an initial denaturation step at 94 °C for 2 min, and then 30 cycles of the following sequence: 94 °C for 20s, 59 °C for 30s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Amplifications were carried out in a thermocycler MyGene L Series Peltier Thermal Cycler (Long Gene). A negative control without DNA was included in each PCR. Reaction products were detected using 1.5% agarose in 1× TAE buffer, with GelRed™



**Figure 1** Macroscopic lesions observed in a dairy calf affected by polyarthritis associated with isolation of *Mycoplasma leachii*. A: Fibrinous carpal joint. B: (1) tenosynovitis of carpi radial extensor; (2) carpal joint.

10 000 $\times$  (Biotium, Inc.) added at a final concentration of 1 $\times$  and visualized in a UV light transilluminator ( $\lambda$  255 nm) (DNR, Bio-Imaging Systems).

The main findings at necropsy were: emaciation, pale mucous membranes and subcutaneous tissues, fibrinous polyarthritis and synovitis of tendon sheaths (Fig. 1). Mild hepatomegaly with intrahepatic cholestasis and biliary vesicle with thick bile was observed in the abdominal cavity. The synovial fluid from the calf in Case 1 was yellow, turbid with fibrin floccules and only a small amount was obtained, despite great enlargement of the joint. No microbial forms were observed by Gram staining from fluids and organs and the presence of inflammatory cells was observed by Giemsa staining. No significant bacterial growth was observed on blood agar, while typical *Mycoplasma* colonies ("fried-egg" appearance) were observed in Modified Hayflick's agar, using a stereo-microscope at 15–25 $\times$  magnification, after 3–6 days of incubation from the following samples: synovial fluid pre mortem, lymph nodes and joint capsule post-mortem from the calf in Case 1 and from synovial fluid and mandibular abscess content from the two calves in Case 2. All isolates were identified as *Mycoplasma* spp. by the PCR technique, but no specific amplification of the fragment corresponding to *M. bovis* was obtained. The 16S-23S rRNA gene sequence obtained from the first isolate from Case 1 showed 99% identity with *M. leachii*. A fragment of 266 bp corresponding to *M. leachii* was amplified from all 5 isolates.

The isolation of *M. leachii* both from pre mortem and post mortem samples added to the lack of bacterial growth strongly suggests that this organism was responsible for the observed pathological condition. However, since no virus isolation was attempted, a contribution of a viral agent as a

primary pathogen cannot be ruled out. Clinical signs and pathological findings described in this case were compatible with previous descriptions of polyarthritis in calves caused by *Mycoplasma* sp. bovine group 7 (MBG7) in Australia<sup>8</sup>; particularly those related to the involvement of several joints and the fibrinous character of secretions.

*Mycoplasma* sp. bovine group 7 of Leach was early described as a cause of arthritis and mastitis in cattle in Australia<sup>5</sup>. Over the last 50 years, several reports on the isolation of this organism both from joints and other anatomic sites (including stomach contents and lungs from aborted fetuses), individual and bulk tank milk have been documented in Australia, while there are only few descriptions of its presence in other cattle-rearing countries<sup>6,8</sup>.

*Mycoplasma* sp. bovine group 7 was classified as one of the six recognized members of the *M. mycoides* cluster. However, serological assays used for species differentiation within this cluster yielded cross reactions between MBG7 and other members of the group<sup>6</sup>. Further phylogenetic studies within members of the *Mycooides* cluster suggested that MBG7 was most closely related to *Mycoplasma capricolum*<sup>6</sup> and recent studies demonstrated a distant phylogenetic position of MBG7 compared with other *Mycoplasma* species. These features, in addition to the distinct biological, phenotypic and genotypic characteristics displayed by members of this group, led to the proposal to elevate this taxon to the new species *M. leachii*<sup>10</sup>.

In the present study, *M. leachii* was isolated both pre and post mortem from the calf in Case 1 and from samples from two calves in Case 2. Although no attempts were made to determine the clonality of isolates, previous studies reported that isolates of *M. leachii* that were obtained from

multiple sites of different animals from the same herd during an outbreak and 18 months after the initial episode belonged to the same clone<sup>6,8</sup>, indicating persistence within the herd. In addition, the isolation of this organism from joints and a lymph node was in accord with a previous description<sup>8</sup> and indicates its capability of migrating and establishing infections in different organs within an animal. Although no previous reports of isolation of *M. leachii* from mandibular abscesses were found, the isolation of other species of *Mycoplasma* from subcutaneous decubital abscesses had been reported<sup>9</sup>.

In conclusion, there are few reports regarding *Mycoplasma* isolation associated with cattle disease in Argentina. In the present study, the isolation of *M. leachii* from diseased cattle in a dairy herd was reported for the first time. This report, as well as studies reporting the isolation of different *Mycoplasma* species in cattle in Argentina<sup>4,12</sup> underscores the importance of the inclusion of *Mycoplasma* testing in routine diagnostic laboratories and the need of conducting epidemiological studies to increase knowledge on the prevalence and distribution of these infectious agents in cattle.

### Conflict of interest

The authors declare that they have no conflicts of interest.

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