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**Original Article**

# Detection of Viable *Mycobacterium bovis* in Lungs and Livers Sold in Butchers' Shops in Buenos Aires, Argentina

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## Abstract

Although *Mycobacterium bovis* is the major etiological agent of tuberculosis in bovines, it can infect other mammalians. Previously reported cases of tuberculosis caused by *M. bovis* in cats from the Autonomous City of Buenos Aires (CABA) led to the conclusion that the main source of infection for these felines was the ingestion of raw bovine lungs. Thus, for this study, we collected samples of bovine viscera from butchers' shops of the Greater Buenos Aires (GBA) and the CABA to assess presence and viability of these mycobacteria in bovine lungs (including the lymph nodes) and livers. We analyzed 216 different samples and obtained 5 isolates of *M. bovis* (4 from lungs and 1 from liver) by culture analysis. We also confirmed the presence of different isolates by polymerase chain reaction, spoligotyping, and MIRU-VNTR assays. The results obtained in this work emphasizes the need of social education for food hygiene, and to change the habit of feeding pets with raw viscera, which carries the risk of epizootic and zoonotic transmission. Moreover, control and eradication programs of bovine tuberculosis should be strengthened and improved.

**Keywords:** *Mycobacterium bovis*, culture, molecular typing, butchers' shop

## Introduction

Bovine tuberculosis is caused mainly by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC). In 2016, its prevalence in Argentina was 0.3%, estimated on the basis of the confiscation rate in federal slaughterhouses during bovine inspection and by the detection of lesions, according to the epidemiologic surveillance of the National Control and Eradication of Bovine Tuberculosis Program (SENASA, 2016). However, in most cases, these lesions are not confirmed by culture. Nevertheless *M. bovis* is still being detected although in low proportions, in other farm animals, pets, wild animals, and even in humans.

Cats are more susceptible to mycobacterial infections such as *M. bovis* (Underwood *et al.*, 1999; Gunn-Moore, 2014). They are infected by the ingestion of raw lungs and milk without a thermal treatment (Fernández and Morici, 1999; Underwood *et al.*, 1999). Although it has been suggested that the risk of cat to human transmission of *M. bovis* may be very low, a report in England revealed the first documented case (https://www.gov.uk/government/news/cases-of-tb-in-domestic-cats-and-cat-to-human-transmission-risk-to-public-very-low). Moreover, molecular evidence shows a potential link between *M. bovis* spoligotyping patterns (spoligotypes) obtained from cats and those isolated from bovines in our country. This finding strengthens the hypothesis that cats become infected mainly by eating raw viscera (Barandiaran *et al.*, 2017).

Additionally, the zoonotic risk increases when domestic animals are infected, because of the close relationship between humans and pets. Because *M. bovis* infection might cause lesions that are imperceptible on macroscopic evaluation, some infected organs may be sold despite having viable bacilli.

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The aim of this study was to assess the food contamination risk because of the presence of viable *M. bovis* in viscera from different markets of the Autonomous City of Buenos Aires (CABA) and the Greater Buenos Aires (GBA). The selected samples were bovine lungs (including the lymph nodes) and livers and the presence of the bacilli were confirmed by molecular tools.

## Materials and Methods

### *Samples*

Six establishments from the (GBA) and one from the CABA were selected by convenience. The visits to each establishment were made on different days.

A total of 216 complete organs, including 210 lungs (with their respective mediastinal lymph nodes) and six livers were studied (Table 1). One hundred thirty-seven lungs were collected from a slaughterhouse after passing food inspection (Table 1). The remaining 73 lungs and all the livers were bought in different butchers' shops.

All the viscera were inspected and palpated to find any distortion of the tissue that may indicate granulomatous lesions (GL). Transversal cuts were made 1 cm apart on the complete organ and a sample was taken when GL were found. If GL were not found in the organ, normal tissue and mediastinal lymph nodes were also sampled. A detailed inspection of the complete organs was performed individually to prevent cross contamination during manipulation in the laboratory.

### *Culture*

The sampled tissue was cut into small pieces with scissors and put into a sterile bag with 20 mL of double-distilled sterile water. Mechanical maceration was performed using a *Masticator* (IUL Instruments, Spain). Petroff's decontamination method with NaOH 4% was performed to culture the samples in Stonebrink media (CEPANZO, 1988) in triplicate. Cultures were held at 37°C for 60 d and evaluated on a weekly basis.

### *Detection by polymerase chain reaction*

Colonies obtained by culture were collected with a sterile disposable loop and suspended in 200 $\mu$L of double-distilled sterile water in a 2 mL-tube with a screw cap. The tube was incubated at 95°C for 30 min to lyse the bacilli and release the DNA according to Supply *et al.* (2000) with modifications. After that, it was centrifuged at 12,000 rpm for 10 min. The supernatant was collected in a sterile tube and 10 $\mu$L were used as polymerase chain reaction (PCR) template. For the identification of mycobacteria of MTC, the IS*6110* target sequence was amplified by using Touch-Down cycling, previously described by Zumárraga *et al.* (2005).

Additionally, to confirm that the *M. bovis* strains isolated were wild types, we analyzed the presence of *esx*A and *esx*B genes, which codify the proteins Esat-6 and Cfp-10 deleted in vaccinal *M. bovis* Bacillus Calmette-Guérin (BCG) strains, respectively. For the amplification of *esx*A, the esxAfLM (5′GAGAGATCTCATGACAGAGCAGCAGTGGAATTTC3′) and esxArLM primers (5′GTTGGATCCTGCGAACATCCC AGTGACG3′) were used. This pair of primers amplifies a 288 bp fragment. For the amplification of *esx*B, the esxBf (5′TGACAACAGACTTCCCGG3′) and esxBr (5′CGATACCCGCGAAATTC3′) primers were used. These primers

TABLE 1. SAMPLES INFORMATION

| *Region* | *Establishments* | *Organs* | *No. of samples* | *No. of samples with lesions* | *No. of* Mycobacterium bovis *isolates* | *Spoligotypes (SB no.)* | *MIRU-VNTR patterns* | *Observations* |
|---|---|---|---|---|---|---|---|---|
| CABA | 1-Butcher's shop | Lungs | 8 | 0 | 0 | | | |
| GBA | 2-Butcher's shop | Lungs | 9 | 0 | 1 | SB0130 | 232214253322 | |
| | 3-Butcher's shop | Lungs | 37 | 9 | 0 | | | 1 GL; 3 lymph nodes enlargement |
| | 4-Slaughterhouse | Lungs | 137 | 29 | 1 | SB0140 | 233324253322 | 15 GL |
| | 5-Butcher's shop | Lungs | 4 | 0 | 0 | | | |
| | 6-Butcher's shop | Liver | 6 | 5 | 1 | SB0120 | 232314251212 | Internal GL in liver; 3 lymph nodes enlargement |
| | | Lungs | 15 | 1 | 2 | | | |
| TOTAL | 6 | | 216 | | 5 | | | |

The results of culture and molecular typing are also shown. Each establishment were identified with a number (1–6).
CABA, Autonomous City of Buenos Aires; GBA, Greater Buenos Aires; GL, granulomatous lesion.

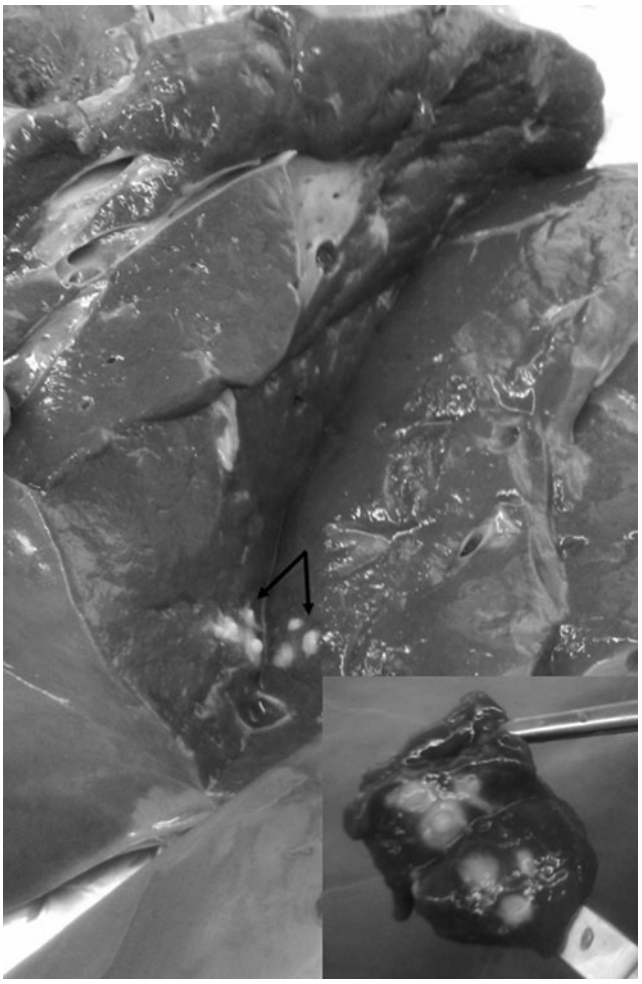

**FIG. 1.** Granulomatous lesions detected in a liver. Multifocal nodular granulomatous lesion with liquefactive necrosis. The organ did not evidence enlargement or abnormalities detectable during inspection.

amplify a 450 bp fragment. For both genes, the reaction was carried out at 95°C for 2 min (1 cycle), followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 30 s at 72°C, and a final elongation at 72°C for 5 min (Encinas, *et al.*, 2018).

Molecular typing. Molecular typing by spoligotyping was conducted as previously described by Kamerbeek *et al.* (1997). Materials such as primers and membrane with bound probes were provided by *Mapmygenome* (Hyderabad, India). The spoligotypes were identified at the *M. bovis* Spoligotype Database (www.Mbovis.org), which is currently managed by VISAVET Health Surveillance Centre of Universidad Complutense Madrid. Molecular typing was completed with the amplification of the 12 MIRU-VNTRs (loci 2-4-10-16-20-23-24-26-27-31-39-40) according to Supply *et al.* (2000).

## Results

By evaluating 216 samples, we obtained five IS*6110* PCR positive isolates from five different organs from GBA. One of them came from a liver and the remaining four positive isolates belonged to lungs and lymph nodes. Four of the positive samples were taken from butchers' shops (three lungs and one liver) and one from a slaughterhouse (lung). The liver presented a GL but this lesion was imperceptible until the organ was sliced (Fig. 1).

From all the organs collected in the slaughterhouse, 29 showed different lesions such as congestion, lymphadenitis, bronchopneumonia foci lesions, and fibrosis, but only 15 exhibited GL and small nodular lesions. However, we could only obtain a *M. bovis* isolate from one of these organs. The remaining four organs sold in butchers' shops, from which the other four isolates were obtained, did not show any GL that may indicate tuberculosis.

Spoligotyping confirmed that all the isolates were *M. bovis* and identified three different spoligotypes: SB0120 ($n=3$), SB0130 ($n=1$), and SB0140 ($n=1$). MIRU-VNTR detected also three different patterns: 232314251212; 232214253322; and 233324253322. Each MIRU-VNTR pattern was related to each different spoligotype.

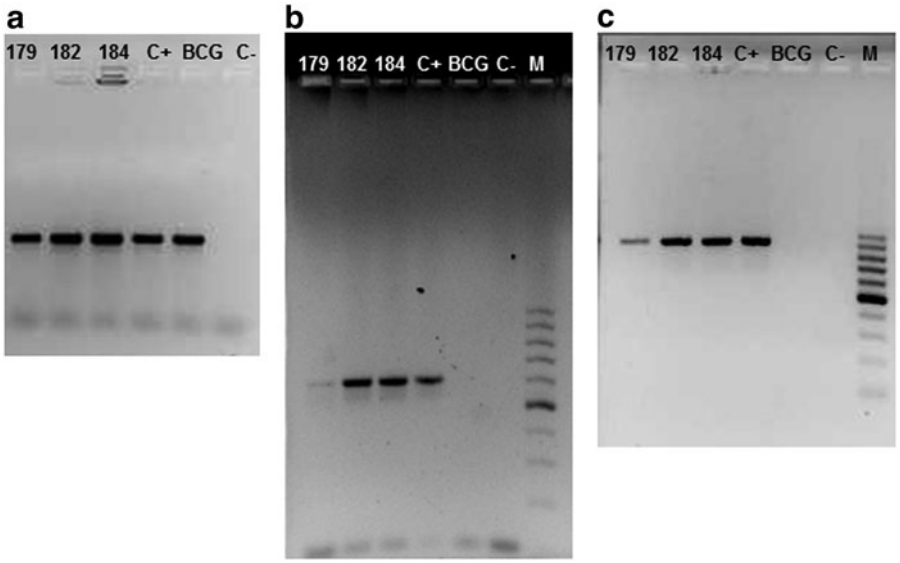


**FIG. 2.** PCR results of IS*6110* **(a)**, *esx*A **(b)**, and *esx*B **(c)**. The number of *Mycobacterium bovis* isolates analyzed is 179; 182; and 184. C+: Positive control, wild-type *M. bovis* strain. C−: Negative control, water. BCG: *M. bovis* BCG Pasteur strain. M: 100 bp DNA Marker (Promega Corp.). BCG, Bacillus Calmette-Guérin; PCR, polymerase chain reaction.

Considering that spoligotype SB0120 is present in both wild-type and vaccinal BCG strains, a deeper analysis based on MIRU-VNTR was performed confirming that the three *M. bovis* isolates were wild-type strains (Table 1). Additionally, these data were confirmed with a specific PCR for the *esx*A and *esx*B genes (absent in all BCG strains) (Fig. 2).

## Discussion

The results presented in this study confirm the presence of viable *M. bovis* strains in bovine organs sold in butchers' shops, evidencing escapes from the routine slaughterhouse inspections mainly due to the inherent characteristics of the disease. In early infections, the incipient development of the lesions can go undetected during the manual and visual inspection, even if the inspection at the slaughterhouse is exhaustive. Accordingly, in this study 1.8% (4/216) of the organs without lesions were infected with viable *M. bovis.*

These results match those obtained by de Kantor *et al.* (1987), where the authors describe that 2.8% (5/178) of the inspected organs that were not condemned after slaughterhouse inspection were later confirmed as *M. bovis* by culture. Although our study is preliminary and limited to a restricted area of Buenos Aires province, the prevalence rate described above suggest an underestimation of the disease.

With regard to the molecular epidemiology of the *M. bovis* isolates, spoligotype SB0140 is most frequently obtained from isolated studies in Argentina (46%), followed by SB0130 (12%), whereas SB0120 represents only 4.9% (Zumárraga *et al.*, 2013). In the case of statistics for the GBA, these spoligotypes represent 40%, 8%, and 4%, respectively, from the total of isolates studied between 1994 and 2016 in Buenos Aires province ($n = 390$) (unpublished data). In the present study, three samples presented SB0120 spoligotype, which is identical to the *M. bovis* BCG vaccine strain. However, using MIRU-VNTRs, these strains could be differentiated from BCG. Additionally, the presence of *esx*A and *esx*B genes was assayed, both of which are deleted in *M. bovis* BCG strains, confirming that these isolates correspond to wild-type strains. In cats, the spoligotype most frequently obtained in Argentina is SB0140, as it is in bovines.

Between 1998 and 2006, 19 isolates of *M. bovis* from cats living in the CABA were typed by spoligotyping (Zumárraga *et al.*, 2009). In one of the reported cases, the owner of a cat with tuberculosis suffered from septic arthritis of the glenohumeral joint, caused by *M. bovis*, and she lived with 20 other cats that she fed daily with bovine lungs bought in a butcher's shop in CABA (Colmegna *et al.*, 2004). This suggests a possible epizootic transmission between cats and bovines when cats are fed raw offal. However, more appropriate techniques such as MIRU-VNTR should be considered to establish those epidemiological links.

Recently, a rare and noteworthy pulmonary TB disease due to *M. bovis* has been reported in a patient who worked as a butcher in a slaughterhouse, who developed a cutaneous granulomatous inflammatory reaction on the dorsal side of his hand (Mertoglu *et al.*, 2018). Thus, the manipulation of bovine organs such as lungs and livers by humans, represents an additional risk factor as they might contain viable mycobacteria, with the associated health concern for zoonotic transmission.

In this study, lungs and livers were assayed to detect viable zoonotic *M. bovis* strains taking into account that livers are consumed by people in Argentina and lungs are mainly used for feeding pets. In the first case, the risk to acquire an infection by consumption of an infected liver is low because the organ is previously cooked, whereas lungs dispensed to pets are generally given raw. However, the manipulation of raw organs represents a possible source of *M. bovis* infection for both, consumers and pet owners.

Food inspection is still a reliable and economical technique to identify tuberculosis and serves as a protection before consumption by humans and pets. Our findings lead to reinforce social education strategies for food hygiene practices to avoid the risk of epizootic and zoonotic transmission of bovine tuberculosis. Moreover, the results raise the need to perform a more exhaustive manual and visual inspection in slaughterhouses with complementary methods to detect potential sources of infection.

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## Disclosure Statement

No competing financial interests exist.

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