# Isolation and molecular characterisation of *Mycobacterium bovis* from raw milk in Tunisia

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## Abstract

**Background:** Consumption of raw milk and unpasteurized dairy products is common in Tunisia where bovine tuberculosis remains enzootic. We herein investigated the frequency of *M. bovis* isolation from raw milk.

**Methods:** Three hundred and six milk samples collected from 102 infected cows in different Tunisian regions were analysed. *M. bovis* isolates were further characterized by spoligotyping and variable number tandem repeat typing.

**Results:** A total of five (4.9 %) *M. bovis* strains exhibiting three different genotypes were isolated.

**Conclusion:** This study demonstrates that consumers of raw milk or derivatives in Tunisia are at high risk of zoonotic infection with *M. bovis*.

**Keywords:** *Mycobacterium bovis*; Tuberculosis; Raw milk, Zoonosis

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## Introduction

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* is a widespread zoonosis of a potential health hazard. Unpasteurized milk and dairy products prepared domestically by fermentation constitute the main source of human *M. bovis* exposure[1]. These types of products are still consumed in Tunisia where BTB remains enzootic. A national program for BTB control hs been implemented in Tunisia since 1984[2]. Nevertheless, the disease continues being prevalent mainly in the private sector which owns more than 70% of the cattle livestock[3]. Movement of infected cattle may be one of the major constraints in the control strategy in this sector. The aim of the present study was to show the frequency of *M. bovis* isolation from raw milk of infected cows in Tunisia in order to estimate the risk of food-borne transmission of *M. bovis*.

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## Methods

### Sampling

Milk sampling was performed in 9 private farms recognized as BTB infected localized in three Tunisian governorates including Tunis (North region), Sousse and Monastir (Central regions), from October 2005 to January 2006. Farms were categorised according to the herd size: A (<5 cattle), B (from 5 to 20 cattle) and C (>20 cattle). Samples (N= 306) were collected during milking from 102 Pie-Noire and Pie-Noire Holstein cows that were positive for the single cervical intradermal tuberculin test (SCITT), whether showing or not the clinical signs of tuberculosis and mastitis. We collected three specimens/animal with an interval of 15 days between each sampling. One *M. bovis* strain isolated from a human sample at the Microbiology and Immunology Laboratory, Farhat Hached Hospital, Sousse, Tunisia was included as positive control.

### Bacteriological and molecular analysis

All samples were processed for *M. bovis* isolation in the veterinary research institute of Tunisia as previously described[4]. Briefly, a volume of 10 ml of raw milk was decontaminated with 4% sodium hydroxide and then neutralized using concentrated hydrochloric acid. Suspensions were then centrifuged at 3 000 g for 30 min and the sediment was

inoculated onto Lowenstein-Jensen and Coletsos media and incubated at 37°C for 8 weeks. *M. bovis* characterization was performed using the Ziehl-Neelsen staining, culture characteristics (growth rate, colony morphology), IS*6110* and RD4 based-PCR, spoligotyping and variable number tandem repeat (VNTR) typing[5,6,9,10]. Spoligotype patterns were named according to the international spoligotype database nomenclature (http://www.mbovis.org).

### Ethics

The experiments comply with the current laws of the country in which they were performed.

## Results

Of the 102 SCITT positive cows included in our study, 5 (4.9%) were detected as shedders *M. bovis* in the milk. These cows belong to the three farm categories i.e. category A (1 cow), B (1 cow) and C (3 cows) (table 1). In addition, 6 nontuberculosis mycobacteria were isolated and only one strain was identified as *M. flavescens* using the commercial kits Genotype Mycobacteria CM and AS (data not shown). The presence or absence of mycobacteria could not be confirmed for 28 of the raw milk cultures because of overgrowth by other contaminant microorganisms.

**Table1: Geographical origin, herd size and sampling size from the nine farms included in this study**

| Origin | Governorate | Category [a] | No. of cows | No. of milk samples | No. of *M. bovis* isolates |
|---|---|---|---|---|---|
| Farm 1 | Monastir | B | 2 | 6 | 0 |
| Farm 2 | Monastir | A | 2 | 6 | 1 (Mb [b]1) |
| Farm 3 | Monastir | B | 4 | 12 | 1 (Mb2) |
| Farm 4 | Monastir | A | 2 | 6 | 0 |
| Farm 5 | Monastir | A | 2 | 6 | 0 |
| Farm 6 | Monastir | C | 7 | 21 | 0 |
| Farm 7 | Sousse | C | 6 | 18 | 0 |
| Farm 8 | Tunis | C | 55 | 165 | 2 (Mb3, Mb4) |
| Farm 9 | Tunis | C | 22 | 66 | 1 (Mb5) |
| Positive control | Sousse | - | - | - | 1 (Mb6) |

[a] Farms were categorised according to the herd size: A (<5 cattle), B (from 5 to 20 cattle) and C (>20 cattle)

[b]Mb= *M. bovis*

To investigate the genetic diversity and relatedness of the 6 *M. bovis* isolates (5 cattle and 1 human isolates), genotyping using spoligotyping and VNTR typing techniques was carried out. A total of 3 different spoligopatterns were obtained (table 2). One spoligotype pattern (strain Mb1) had not been previously reported on the *M. bovis* database, and it was therefore designated SB1200. In addition to the deletion of spacers 3, 9, 16 and 39 to 43, characteristic features of cattle isolates[9], this type was characterized by the lack of spacers 28 and 29. The VNTR analysis further distinguished 4 different patterns. The human *M. bovis* strain (Mb6) included in this study as positive control showed the same spoligopattern (SB1093) and VNTR profile (5-4-2-4-3-2-10-3-11-6) as strain Mb2 isolated from raw milk. This strain was isolated from a cutaneous lesion of farmer women strongly suggesting an occupational exposure to *M. bovis*.

**Table 2: Spoligotypes and variable number tandem repeat (VNTR) profile of the *M.bovis* isolates characterized in this study**

| M. bovis isolate | Spoligo-pattern designation[a] | Spoligopattern[b] Spacer 1 — 43 | VNTR profile[c] |
|---|---|---|---|
| Mb1 | SB1200 | 1101111101111110111111111111001111111100000 | 5-8-4-4-3-2-12-3-7-6 |
| Mb2 | SB1093 | 1100111101111110111101111111111111111100000 | 5-4-2-4-3-2-10-3-11-6 |
| Mb3 | SB1093 | 1100111101111110111101111111111111111100000 | 5-4-2-4-3-2-12-3-13-6 |

Continuation of table 2

| M. bovis isolate | Spoligo-pattern designation[a] | Spoligopattern[b] Spacer 1 ——— 43 | VNTR profile[c] |
|---|---|---|---|
| Mb4 | SB1003 | 11011101011111101111111111101111111111100000 | 5-4-4-4-3-2-12-4-15-5 |
| Mb5 | SB1003 | 11011101011111101111111111101111111111100000 | 5-4-4-4-3-2-12-4-15-5 |
| Mb6 | SB1093 | 11001111011111101111011111111111111111100000 | 5-4-2-4-3-2-10-3-11-6 |

[a] www.mbovis.org

[b] 1= spacer present, 0= spacer absent

[c] The VNTR profile is defined by the number of tandem repeats in ETR-A, -B, -C, -D, -E,-F, QUB-11a, -11b, -3336 and -26 loci consecutively.

## Discussion

Unfortunately, during the study period we did not have the opportunity to follow up the cows excreting *M. bovis* in the milk at post-mortem to further check udders and mammary lymph-nodes. This would be interesting for examining the correlation between *M. bovis* excretion in milk and the presence or absence tuberculous lesions. The fact that *M. bovis* was isolated from both small (category A) and large (category C) farms is of considerable importance particularly for category A farms. Indeed, the small quantity of produced milk may not be sold to dairy industry for pasteurization, but sold at retail and may be consumed raw or used for producing fermented dairy products. The exact relative contribution of *M. bovis* in the Tunisian human population is unknown. Nevertheless, twelve human extrapulmonary tuberculosis cases due to *M. bovis* including one multi-drug resistant strain have previously been reported (Slim-Saidi L. unpublished data). The consumption of raw milk or derivatives was mentioned in six (60 %) out of ten cervical lymphadenopathy cases. Our data support the premise that raw milk is a potential source of *M. bovis* transmission in Tunisia and thus particular vigilance is necessary. The frequency of *M. bovis* isolation reported here is higher than that reported in other developing countries[7,8]. The reason for this result may be due in part to the sample origin. Indeed, we collected milk samples only from positive SCITT cows while Srivastava el al[8] included tuberculin negative and apparently healthy animals and Leite et al[7] collected milk samples from retail markets.

Spoligotyping and VNTR analysis revealed the heterogeneity of the six *M. bovis* isolates (five cattle and one human isolate). Indeed, three spoligopatterns and four VNTR profiles were obtained (table 2). So far, however, it is not possible to make any epidemiological link between the different strains since our collection is limited in size and geographical representation. In addition, no data showing the genetic diversity of *M. bovis population* in Tunisia are available. According to the spoligotype database, the molecular characteristics of *M. bovis* strains described here are comparable, more likely, to molecular types of *M. bovis* strains isolated in European countries rather than those in African countries including neighbouring Algeria[11]. This finding needs to be further elucidated by larger molecular investigations.

## Conclusion

As long as BTB remains enzootic in Tunisia, consumers of raw milk and unpasteurized dairy products may be exposed to *M. bovis* infection. Therefore, control campaigns in cattle in addition to public health education on the risk associated with the consumption of raw milk and fresh dairy products should be accentuated. Molecular epidemiological studies would be very helpful to increase our knowledge of *M. bovis* dissemination in Tunisia.

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