Original Article
Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.5327/fst.484
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Molecular analysis of the *Mycobacterium tuberculosis* complex in refrigerated raw milk and geospatial distribution in Brazilian small farms

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Abstract

This study reports the investigation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in 102 milk samples from lactating cows, collected directly from cooling tanks of small family farms located in eight municipalities in the state of São Paulo, Brazil. Conventional polymerase chain reaction and real-time polymerase chain reaction techniques were used, as well as spatial analysis. All milk samples tested were negative for *M. tuberculosis* and *M. bovis*. Nevertheless, constant epidemiological surveillance of these pathogens is important because raw milk is still widely consumed and has great infectious potential, besides its use in the production of dairy products such as cheese and butter, which are important sources of income for small producers, thus posing risks to public health and farmers' income.

Keywords: *Mycobacterium tuberculosis* complex; bulk tank milk; bovine; molecular; PCR; DNA.

Practical Application: Bulk tank milk may be a source of contamination by *Mycobacterium tuberculosis* and *Mycobacterium bovis.*

1 INTRODUCTION

Tuberculosis (TB) is an infectious disease with global distribution and chronic evolution that can affect humans, domestic animals, and wildlife (Bolaños et al., 2017). The species of greatest relevance in the context of public health are *Mycobacterium tuberculosis* (the most common cause of TB in humans) and *Mycobacterium Bovis* (mainly responsible for zoonotic TB), which correspond to approximately 3.1% of all TB cases globally (Collins et al., 2022; Kader et al., 2023). Transmission can be horizontal (occurring from cattle to cattle), interspecies (from cattle to wild animals and humans), and vice versa (through air and ingestion of water and food contaminated by aerosols generated after excretion into the environment) (Rua-Domenech, 2006; Smaniotto et al., 2019).

In the case of humans, another form of contagion that is gaining prominence is the ingestion of raw milk that has not been pasteurized, boiled, or processed at ultra-high temperature (UHT). In Brazil, 20 to 30% of cow's milk is sold without inspection, which increases the potential for the transmission of pathogens. Also, many farm families consume raw milk due to deep-rooted cultural habits and because it is cheaper to take milk from the bulk tank than to buy pasteurized milk in retail stores. In addition, some consumers believe that raw milk has higher nutritional value than pasteurized milk and that this process reduces its quality (Deneke et al., 2022). The introduction of raw milk into dairy processing poses a significant risk of contamination of dairy products that can lead to human exposure to pathogenic bacteria.

Therefore, correct diagnosis is essential for tallying statistics and the subsequent formulation of measures to combat the disease. Currently, microbiological culturing of *M. bovis* in media containing pyruvate is considered the gold standard test for the diagnosis of TB. However, it has certain limitations, including slow delivery of results and low sensitivity (Singh et al., 2022). In this light, several studies have suggested that the action of *M. bovis* in humans is underestimated. Notification statistics are typically reported as a proportion of the total number of human TB cases and are usually from small-scale studies or based on retrospective data reviews. Hence, the statistics do not represent the real picture of the disease (Assi et al., 2021; Devi et al., 2021; Olea-Popelka et al., 2017). The use of molecular techniques provides a rapid alternative to culturing and allows for the collection of epidemiological data, such as pathogen

Received: May 11, 2025.
Accepted: Jun. 12, 2025.
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Conflict of interest: nothing to declare.
Funding: This research was supported by the Sao Paulo Research Foundation (FAPESP), Brazil, grant numbers 2020/09409-2 and 2022/08331-5; and the Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarship grant.

population levels, evolution and graphical geodistribution, and identification of new species.

In most of the affected patients, there is no identification of the species of mycobacteria causing TB, increasing the risk of inadequate treatment (Olea-Popelka et al., 2017). *M. bovis* is naturally resistant to pyrazinamide, one of the four drugs used in the standard anti-TB treatment regimen. In addition to the impacts on public health, bovine TB also affects economic sectors. Its estimated impacts amount to three billion dollars a year worldwide (Ramanujam & Palaniyandi, 2023). These losses result from reduced animal production (decreased weight gain and lower yield of meat or milk), as well as the need to sacrifice positive animals and totally or partially condemn carcasses. The degree of impairment is inversely proportional to the price paid for the animals (Smaniotto et al., 2019).

Since 2001, the control and eradication of bovine TB in Brazil have been carried out by the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2006), whose main function is to reduce the prevalence and incidence of TB and brucellosis and their impacts on human and animal health. This is achieved through prophylactic actions involving diagnostic and sanitation methods in cattle herds under active health surveillance, in addition to creating a significant number of certified or monitored properties that offer consumers products with low health risks (Oliveira, 2019). However, the low certification rate of properties as free of brucellosis and TB is due to the low economic return provided to producers. This factor, combined with underreporting, the lack of awareness among producers themselves, and the dearth of well-trained veterinarians, poses obstacles to the control of these diseases (Oliveira, 2019).

Based on this scenario, zoonotic TB is a serious public health problem. Therefore, cooperation among the veterinary, clinical, epidemiological, and public health sectors is necessary to design and structure TB eradication programs that integrate both human and scientific fronts (Ramanujam & Palaniyandi, 2023). Detailed studies of the causes that aggravate the prevalence of TB on small farms and the regions with the highest incidence of the disease can enable the development of specific actions to combat and raise awareness about the disease. To address this problem, we analyzed the presence of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in milk samples from individual expansion tanks on small family farms using the conventional molecular techniques of polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR), to perform genetic sequencing of positive samples and assess their spatial distribution.

1.1 Relevance of the work

Zoonotic tuberculosis is an underreported disease that requires engagement among the veterinary, clinical, epidemiological, and public health sectors to design and structure programs for its eradication. Dairy farming is an economic activity of great importance in the Brazilian state of São Paulo, where there are still many small family farms. Molecular analyses of the *Mycobacterium tuberculosis* complex and *Mycobacterium bovis* were performed on milk samples from individual expansion tanks of small farms, for the purpose of epidemiological surveillance of this neglected but still present zoonosis.

2 MATERIAL AND METHODS

2.1 Ethical aspects

This study was approved by the Research Ethics Committee (CEP) of the Botucatu Faculty of Medicine (FMB – UNESP) – Certificate 1390/2021.

2.2 Cow milk samples from expansion tanks

The milk samples were properly stored in the Animal Health Laboratory of

APTA Centro-Oeste – Bauru, in a freezer at −20°C. The samples were obtained from 102 individual expansion tanks located at different farms in eight municipalities in the central-west region of the state of São Paulo, with one milk sample per property: Anhembi (13 samples), Avaré (47 samples), Arandu (4 samples), Bofete (7 samples), Botucatu (2 samples), Bauru (3 samples), Cerqueira Cesar (10 samples), and Pardinho (16 samples). Figures 1 and 2 show maps produced using the QGIS software, depicting the state of São Paulo and the municipalities where the samples were collected.

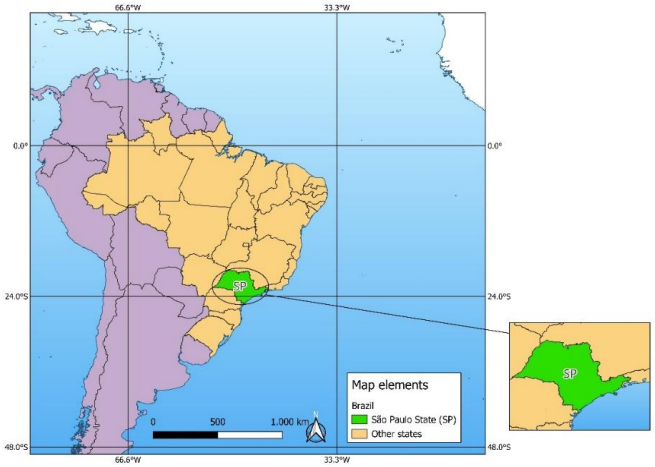

Figure 1. Map of the state of São Paulo, Brazil.

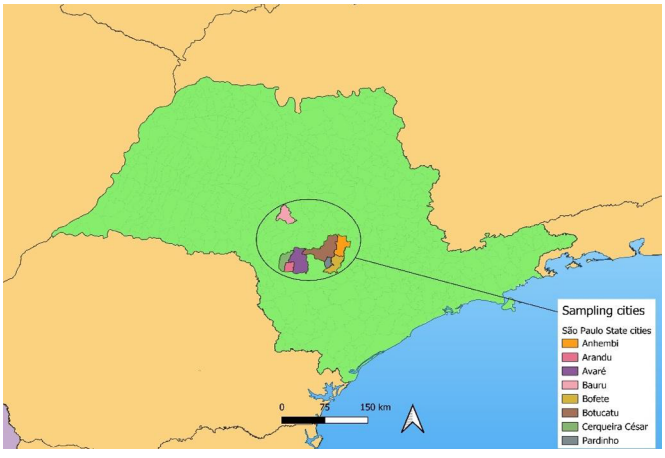

Figure 2. Map of the state of São Paulo and the location of the municipalities of the properties where milk samples were collected.

All milk samples in the tanks had not yet undergone pasteurization (raw milk) and were intended to be sent to dairy producers in the region studied so that they could be pasteurized.

According to the relevant regulations, cooling tanks must have a maximum temperature of 4°C and must not store milk from milkings carried out more than 48 h beforehand.

2.3 Molecular tests

2.3.1 DNA extraction from milk samples

Deoxyribonucleic Acid (DNA) from the milk samples was extracted with the commercial kit GFX Genomic Blood (GE Healthcare®), according to the protocol indicated by the manufacturer, with slight modifications suggested by Cunha et al. (2006).

2.3.2 DNA quantification

We used a NanoVue Plus® spectrophotometer from GE Healthcare Bioscience/UK to verify the amount of DNA in each sample.

2.3.3 Conventional polymerase chain reaction – Mycobacterium tuberculosis complex

We performed molecular identification by conventional polymerase chain reaction (cPCR) for *Mycobacterium tuberculosis* complex (MTC) using the IS6110 target, according to García-Cortés et al. (2021) and Liébana et al. (1996). Initially, we tested the samples for the IS6110 insertion sequence (IS), using the primers described below, which identify samples positive for the MTC:

INS–1 CGTGAGGGCATCGAGGTGGC

INS–2 GCGTAGGCGTCGGTGACAAA

The reactions were performed according to the following conditions: Each 0.2 mL reaction tube received PCR buffer (50 mM KCl, 20 mM Tris-HCl), 0.75 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 µL of Taq-polymerase (Platinum® Taq DNA Polymerase, Invitrogen®), 0.5 µM of each primer, 2 µL of the tested sample, and 17.5 µL of ultrapure water (MIX-PCR). Thus, each tube contained 23 µL of MIX-PCR and 2 µL of the DNA extraction product. cPCR was performed to amplify the 245 bp repetitive fragment, which consists of the amplification of the IS 6110 (IS6110) of the *Mycobacterium tuberculosis* complex (MTC) constituents using the primers INS-1 and INS-2.

Amplification was performed using a Mastercycler Pro gradient thermocycler (Eppendorf®) with cycling according to Hermans et al. (1990) with adaptations. Thus, the cycle began with an initial denaturation step at 94°C for 10 min, followed by amplification for 35 cycles consisting of denaturation at 94°C for 1 min and annealing of the primers of the target DNA at 65°C for 1 min. Then, the temperature was increased to 72°C and was held for 2 min until the extension of the new DNA strand, followed by a final extension cycle of 10 min at 72°C. Afterward, the mixture was refrigerated at 4°C.

The samples were also tested for the molecular identification of *M. bovis* using the oxy R gene, with a final reaction volume of 25 µL.

oxyRMB-1 (5′-GCACGACGGTGGCCAGGCA-3′)

oxyRMB-2.1 (5′-TGGCCGGGCTTCGCGT-3′)

The reactions were performed under the following conditions: each 0.2 mL reaction tube received PCR buffer (50 mM KCl, 20 mM Tris-HCl), 0.75 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 µL of Taq-polymerase (Platinum® Taq DNA Polymerase, Invitrogen®), 0.5 µM of each primer, 2 µL of the tested sample, and 17.5 µL of ultrapure water (MIX-PCR). Thus, each tube contained 23 µL of MIX-PCR and 2 µL of the DNA extraction product. The amplification generated a 270 bp fragment. The cycling process occurred initially at 95°C for 12 min, followed by 30 two-step cycles including denaturation at 94°C for 45 s and annealing plus extension at 70°C for 90 s (Espinosa de los Monteros et al., 1998).

2.3.4 Controls

As positive controls, we used *M. bovis* AN5 strains from cultures, kindly provided by the Laboratory of Bioprocesses and Biotechnology for the Diagnosis of Tuberculosis and Diseases of Importance to Health of UNESP – Botucatu. As controls for *M. tuberculosis*, we employed human sputum samples, kindly provided by the Adolfo Lutz Institute of Sorocaba, São Paulo, proven to be positive for *M. tuberculosis* by the qPCR technique according to Flamínio (2019). For the negative control, we used nuclease-free water.

2.3.5 Agarose gel electrophoresis

Aliquots of 10 µL of the amplified samples were homogenized with 2 µL of running buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and 70% ultrapure water) in a horizontal cuvette containing 1X TBE (0.1 M Tris, 0.09 M boric acid, and 0.001 M ethylenediaminetetraacetic acid [EDTA]).

The identification of the amplified products was performed by electrophoresis in 1.5% agarose gel after staining with Syber Safe® (Invitrogen) 0.1 µL/mL. The voltage used was 100 V for 90 min. For the molecular weight standard, the LowRanger 100 bp DNA Ladder (Norgen) was used, and the sizes of the amplified fragments were visually compared against the molecular weight standards of the standard strains employed as positive controls using ultraviolet (UV) light. The images were captured by a Major Science™ system and were documented using the VisionWorksLS software.

2.3.6 Quantitative polymerase chain reaction

The qPCR was performed according to the method described by Flamínio (2019), using primers for the IS IS1081 of

the MTC, whose marker is found in several copies in the *M. bovis* genome. The following system was used for amplification: GoTaq® qPCR Master Mix (Promega, USA), performed with a 7500 Fast Real Time PCR System with 7500 software v.2.3 (Applied Biosystems).

3 RESULTS

The purpose of this study was to investigate the presence of *M. tuberculosis* and *M. bovis* in 102 milk samples from lactating cows, collected directly from individual cooling tanks on small family farms located in eight municipalities in the state of São Paulo. Conventional molecular PCR and real-time PCR were used, and all samples were negative for both species.

4 DISCUSSION

The most reliable diagnostic method for identifying diseases caused by microorganisms is the isolation and identification of the agent. Although microbiological culture is the gold standard for bovine TB, it is recommended to be performed using tissue samples, in association with histopathological examination, which allows for confirmation of the diagnosis. However, these traditional methods for growing microorganisms in laboratory conditions are still inefficient and time-consuming, and the bacteria must be alive for growth to occur. Furthermore, the difficulty of obtaining samples and the scarcity of *M. bovis* in bovine secretions make the use of this technique unfeasible. Thus, PCR techniques, compared with culture and tuberculin analysis, have better specificity and sensitivity to identify infections associated with *M. bovis* in blood, milk, and nasal mucus samples (Flamínio, 2019; Figueiredo, 2008).

Although the techniques used in this study have high sensitivity and specificity to detect *M. bovis* and *M. tuberculosis*, a limitation may have occurred due to the fact that the samples were collected exclusively from expansion tanks and milk can be composed of a complex of microorganisms, making it difficult to detect mycobacteria in this environment. Nevertheless, the primers, as well as the chosen technique, have sensitivity and specificity for the bovine TB agent, as already tested in clinical samples of lymph nodes from carcasses condemned by the Federal Inspection Service (SIF) due to bovine TB, as described by Flamínio (2019). The test is considered positive if at least one copy of *M. bovis* DNA is present in the sample.

Furthermore, the unique composition and properties of milk and dairy products make them excellent growth media for many pathogenic microorganisms besides those of the MTC (Carneiro et al., 2022). This factor constitutes one of the limitations of our study since the large number of mycobacteria in milk samples from expansion tanks of family farms can make it difficult to identify the target pathogen. In view of this, the molecular tests performed in the present study did not detect samples of expansion tank milk positive for *M. bovis* and *M. tuberculosis*. Despite these negative results, the presence of pathogens in unpasteurized raw milk and the intrinsic risk of its sale and consumption cannot be ruled out under any circumstance.

Bezerra et al. (2015) found that, among 502 samples of raw milk collected from both animals and cooling tanks at small dairy farms in the Brazilian state of Rio Grande do Sul, 10 were positive for bacteria from the MTC (1.99%) and 7 were positive for *Mycobacterium avium* (1.39%), indicating potential public health risks associated with the consumption of raw milk.

In contrast, Deneke et al. (2022) observed that even in cases where a considerable proportion of society is aware of zoonotic diseases, the consumption of boiled or pasteurized milk is low. This may be related to the fact that, although people are aware of the risk, immediate symptoms after the ingestion of raw milk do not necessarily occur, and a latent infection may develop that will only cause clinical symptoms later. In addition, unpasteurized raw milk is more convenient and quicker to obtain in rural settings.

In this sense, there is a need for constant epidemiological surveillance of this zoonosis. In the current scenario, underreporting, along with the challenges of diagnosis and accurate differentiation between *M. bovis* and *M. tuberculosis* and weak epidemiological surveillance systems in most countries (especially developing ones), fail to measure the true incidence of zoonotic TB in humans. Most studies conducted have been small-scale and/or based on retrospective data reviews (Devi et al., 2021).

Differentiation of species within a complex is usually based on the results of biochemical or microbiological tests. Cultivation of mycobacteria on selective media remains the gold standard for the detection of *M. bovis* (Carneiro et al., 2022). Test sensitivity is influenced by the stage of infection and disease, while specificity may vary in different settings depending on the presence of cross-reactive organisms (Collins et al., 2022). Furthermore, these tests are cumbersome, have low sensitivity, and are more time-consuming than molecular techniques. The use of molecular techniques to detect *M. bovis* provides a rapid alternative to culture and enables the collection of important epidemiological information that can aid efforts to eliminate the disease from human and animal populations (Carneiro et al., 2022).

Strict hygienic controls involving the handling of dairy cattle, together with pasteurization of milk, have arguably reduced TB transmission in developed countries (Devi et al., 2021).

Therefore, the need for epidemiological surveillance and the preparation of reports at the national and international levels is justified, aiming to clarify the situation of the disease. Research into better diagnostic tools helps accurately identify the species of mycobacteria for immediate treatment or management in animals and humans. Large-scale studies involving different geographic areas and regions are recommended to assess the impact of *M. bovis* infection and the risk of occupational exposure (Devi et al., 2021), as well as the economic impact, caused by the reduction of productivity and expenses for treatment and mandatory disposal of affected animals (Duarte et al., 2019).

5 CONCLUSIONS

Even though the samples tested were negative for *M. bovis* and *M. tuberculosis*, there is an undeniable need to diagnose

these zoonotic agents in raw milk to ensure safe consumption, as well as for its use to make products such as cheese and butter, which are important sources of income for small producers. Studies involving molecular diagnosis can allow large-scale surveillance for epidemiological monitoring in different geographic areas.

ACKNOWLEDGMENTS

We would like to thank the staff of Adolfo Lutz Institute – Sorocaba. We also thank the São Paulo State Research Foundation (FAPESP), Brazil, for the support research grant numbers 2020/09409-2 and 2022/08331-5; and the Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarship grant.

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