Mycobacteria in Minas cheese commercialized in open fairs in São Paulo, Brazil

Mycobacteria em queijos tipo Minas comercializados em feiras-livres de São Paulo, Brasil

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Abstract

Mycobacterium bovis is the causative agent of bovine tuberculosis, a disease that affects dairy herds throughout the Brazilian territory, constituting a neglected zoonosis transmitted by raw milk and its derivatives. In this study, we evaluated the presence of *M. bovis* and other mycobacteria in Minas cheese obtained from open fairs in the city of São Paulo between 2012 and 2013. Samples (n = 133) were decontaminated using hexa-cetylpyridinium chloride and seeded on Stonebrink–Leslie medium. The isolates were submitted to molecular identification by TB Multiplex PCR targeting the 16S rRNA gene and amplicon nucleotide sequencing. From 16 cheese samples (12%), we obtained 26 putative colonies of *Mycobacterium* spp, none of which belonged to any of the *Mycobacterium tuberculosis, Mycobacterium avium*, or *Mycobacterium intracellulare* complexes. Phylogenetic analysis showed that sample sequences were grouped in a clade that includes only non-tuberculous mycobacteria with proximity to sequences obtained from *Mycobacterium novocastrense* (3 sequences), *Mycobacterium holsaticum* (1 sequence), and *Mycobacterium elephantis* (2 sequences). Although no epidemiological evidence was found regarding the importance of oral transmission of mycobacteria in healthy people, their importance in the immunosuppressed population remains uncertain.

Keywords: Mycobacteria. Non-tuberculous mycobacteria. *Mycobacterium bovis*. Minas cheese. Free-trade fair.

Resumo

Mycobacterium bovis é o agente da tuberculose bovina, doença que acomete o rebanho em todo território brasileiro e é uma negligenciada zoonose transmitida pelo leite e seus derivados. Este trabalho avaliou a presença de *M. bovis* e outras micobactérias, em queijo minas meia-cura, obtidos em feiras-livres na cidade de São Paulo, entre os anos de 2012 e 2013. As amostras (n = 133) foram descontaminadas pelo método HPC (hexa-cetyl-pyridinium chloride) e semeadas em meio Stonebrink Leslie. Os isolados foram submetidos à identificação molecular por PCR TB multiplex, pesquisando-se o gene 16S rRNA, e ao sequenciamento nucleotídico. Dezesseis amostras (12%) possuiam 26 colônias sugestivas de *Mycobacterium* spp, mas nenhuma delas pertencia aos complexos *Mycobacterium tuberculosis, Mycobacterium avium* e *Mycobacterium intracellulare*. A análise filogenética mostrou que todas as amostras estavam agrupadas em clados que incluem apenas micobactérias não tuberculosas (MNT), sendo que algumas possuiam proximidade com sequências obtidas de *Mycobacterium novocastrense* (3 sequências), *Mycobacterium hosaticum* (1 sequência) e *Mycobacterium elephantis* (2 sequências). Embora no momento não haja evidência epidemiológica da importância da transmissão oral das micobactérias pra indivíduos saudáveis, sua importância na população imunossuprimida ainda é incerta.

Palavras-chaves: Mycobacteria. Micobactérias não tuberculosas. *Mycobacterium bovis*. Queijo tipo minas. Feiras-livres.

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Introduction

The genus *Mycobacterium* comprises 198 species (PARTE, 2018), including opportunistic, saprophytic, and obligate pathogens (FORBES, 2017). Within the latter group, *Mycobacterium bovis*, which belongs to the *Mycobacterium tuberculosis* complex, is a species with epidemiological importance for humans and animals, as it is the causative agent of zoonotic tuberculosis. Opportunistic and saprophytic mycobacteria, also known as non-tuberculous mycobacteria, are ubiquitous microorganisms found in both soil and aquatic habitats (PFYFFER et al., 2003).

Although the occurrence of zoonotic tuberculosis in humans has declined dramatically in countries that have adopted systematic milk pasteurization (THOEN et al., 2006; BOSE, 2008), it remains important in countries where the disease persists in herds and among human populations that consume raw milk and its derivatives (OLEA-POPELKA et al., 2017). In Brazil, despite the lack of epidemiological data on the involvement of *M. bovis* in human tuberculosis events, it is reasonable to assume that cases are attributable to transmission via the consumption of unpasteurized dairy products, and thus the risk of exposure among the Brazilian population can be evaluated by assessing infection occurrence data in the bovine population and the size of the informal milk market.

Studies conducted in 13 Brazilian states, covering 75% of the country's bovine population, found that the prevalence of tuberculosis ranged from 0.36% in the Federal District to 9.0% in São Paulo (BAHIENSE et al., 2016; BARBIERI et al., 2016; DIAS et al., 2016; GALVIS et al., 2016; GUEDES et al., 2016; LIMA et al., 2016; NÉSPOLI et al., 2016; QUEIROZ et al., 2016; RIBEIRO et al., 2016; ROCHA et al., 2016; SILVA et al., 2016; VELOSO et al., 2016; VENDRAME et al., 2016). Furthermore, the volume of informal milk produced in 2015 accounted for approximately 31% of milk (11 billion liters of a total of 35 billion liters) produced in the country (ESTATÍSTICAS, 2018), which is mainly destined for the manufacture of raw milk products, particularly cheese, due to the ease of production, high added value, and considerable demand (MACIEL et al., 2008).

Elsewhere, countries that control tuberculosis in animals and pasteurize all milk are, nevertheless, still vulnerable to outbreaks of this disease due to the illegal importation of contaminated products brought in travelers' luggage. For example, in New York, between 2001 and 2004, an outbreak of human tuberculosis was associated with the consumption of fresh Mexican cheese made from raw milk. Thirty-five people were affected with one fatality (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2005). In Mexico, approximately 16% of dairy cows are infected with *M. bovis*, and it is estimated that between 30% and 50% of commercialized dairy products are derived from unpasteurized milk (MACÍA PARRA et al., 2011).

The growing concern regarding the continued threat of tuberculosis to human populations, in both Brazil and other countries, has increased research on the prevalence of *M. bovis* in milk and dairy products (LEITE et al., 2003; VIALTA et al., 2003; HARRIS et al., 2007; KONUK et al., 2007). Many researchers, however, have identified only non-tuberculous mycobacteria in these products (LEITE et al., 2003; HARRIS et al., 2007; KONUK et al., 2007; JORDAO JUNIOR et al., 2009; FRANCO et al., 2013; AGADA et al., 2014). Artisan cheeses are widely consumed in Brazil, where the traditional manufacturing techniques typically used by the producers represent a major challenge regarding food safety (MARTINS et al., 2015; MATA et al., 2016).

Artisanal minas cheese is recognized as a cultural heritage of Minas Gerais (MG) and represents an important product of family agriculture in the state. It is made with raw milk using traditional techniques by about 20,000 producers distributed among 74 cities (MINAS GERAIS, 2002, 2017; SULEIMAN, 2018). Specific regulations have been established for this product by the Institute of Agriculture of Minas Gerais (IMA), including herd control for brucellosis and tuberculosis (MINAS GERAIS, 2013). Furthermore, the minimum ripening period at room temperature has been set to 14 days for cheeses from the Araxá microregion, 17 days for cheeses from the Serro microregion, and 22 days for cheeses from Canastra, Cerrado, Campo das Vertentes, Serra do Salitre, and Triângulo Mineiro microregions (MINAS GERAIS, 2017).

Of the 20,000 producers in the state, only 252 establishments are registered at the IMA and are authorized to sell their products in the state of Minas Gerais; of these, only 8 establishments are included in the Brazilian system of inspection of animal

origin products and may be commercialized throughout the national territory (BRASIL, 2017; MINAS GERAIS, 2018, [201-?]). The other producers are unregulated, which means their production is not monitored, and hence may represent a high risk for public health. Moreover, these producers probably practice shorter ripening periods. The cheeses ripened for only a few days are popularly known as Minas half-matured cheeses, and the main consumer market for these products is the city of São Paulo, where the open fairs are the main marketplaces for these cheeses.

In spite of the microbiological hazard associated with the presence of *Mycobacterium* in dairy products, little research on mycobacteria in cheeses has been done, particularly in Brazil. Thus, this study aimed to verify the occurrence of *M. bovis* and other mycobacteria in Minas half-matured cheese sold at open fairs in the city of São Paulo. This cheese should be manufactured with pasteurized milk or, when produced with raw milk, it should be matured for at least 60 days before consumption (BRASIL, 2017). In informal production, however, such practices are generally not adopted, thereby constituting a risk to public health.

Materials and Methods

Samples

Between April 2012 and March 2013, a total of 133 samples of Minas cheese were obtained from open fairs in São Paulo, Brazil. The street markets analyzed in this study were randomly selected from a sample list of existing street markets in São Paulo, kindly provided by the city's Municipal Administration.

Twenty-five grams of each material was homogenized with 225 mL of 0.85% saline solution, and 2 mL of the resulting homogenate was decontaminated using 1.5% hexa-cetylpyridinium chloride for 30 minutes (CORNER et al., 1995), followed by centrifugation at 3,000 × *g* for 20 minutes (KONEMAN et al., 2001). The resulting liquid phase was discarded, and the supernatant (fat) and pellet were again mixed and resuspended in 1 mL of 0.85% saline and 0.05% Tween 80 solution for seeding.

Plating

One hundred microliters of each sample was plated in triplicate on the surface of Stonebrink–Leslie medium (PAN AMERICAN ZOONOSES CENTER, 1972) and incubated at 37°C for 90 days. All the colonies developed on the culture medium were submitted to molecular identification.

TB Multiplex PCR Reaction

DNA was extracted using CTAB n(cetyltrimethylammonium bromide) according to the procedure described by Kremer et al. (1999). For mycobacteria detection and discrimination between *Mycobacterium* spp and *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare* complexes, we amplified 16S rRNA gene fragment, based on the multiplex PCR technique described by Wilton and Cousins (1992), using Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Negative controls were included for each four samples to monitor possible contamination, along with one positive control (*M. bovis* AN5 reference strain). The amplified products were monitored electrophoretically on agarose gels (1.5%), which were stained with 0.5 μg/mL ethidium bromide and visualized under UV light. Positive samples were those that showed fragments of a compatible size (1030 bp) as described by Wilton and Cousins (1992), or those with bands of 372 bp (*M. tuberculosis* complex), 180 bp (*M. avium* complex), and/or 850 bp (*M. intracellulare* complex).

Sequencing Reaction

The amplicons (881 bp) generated in the PCR reactions for the 16S rRNA gene were purified using ExoSap-IT (USB Products Affymetrix, Cleveland, USA) and subjected to sequencing reactions using Big-Dye 3.1 (Applied Biosystems, Carlsbad, USA). Sequences were determined using an ABI-3500 Genetic Analyzer (Applied Biosystems, Carlsbad, USA), according to the manufacturer's instructions.

The nucleotide sequences obtained in this study, along with others that represent different *Mycobacterium* species, including those of the *M. tuberculosis* complex and non-tuberculous mycobacteria, retrieved from GenBank, were aligned using CLUSTAL/W 1.81 software (THOMPSON et al., 1994). A phylogenetic tree was reconstructed based on the neighbor-joining method, using a maximum composite likelihood model for nucleotide substitution. The bootstrap values were generated with 1,000 replicates, using MEGA 6.06 software (TAMURA et al., 2013).

Results and Discussion

Among the 133 cheese samples examined, colonies of *Mycobacterium* spp were obtained from 16 samples (12%), totaling 26 isolates. The results of TB Multiplex PCR indicated that none of the colonies belonged to the

M. tuberculosis, *M. avium,* or *M. intracellulare* complexes, and thus these mycobacterial isolates were classified as non-tuberculous mycobacteria.

From the 26 colonies whose sequencing was analyzed, we obtained 16 sequences that had sufficiently high quality to enable phylogenetic analysis. The sequences generated were deposited into GenBank with the following accession numbers: KY522830–KY522837, KY522841–KY522845, KY522849, KY522850, and KY522853.

Analysis of 16S rRNA gene fragments (Figure 1) showed that the sequences obtained in this study were grouped into a clade encompassing only non-tuberculous mycobacteria, thereby confirming the PCR results. Further phylogenetic reconstruction indicated that there were small phylogenetic distances between sequences KY522850, KY522845, and KY522844 and that of *M. novocastrense* species; between sequences KY522835 and KY522836 and that of *M. elephantis*; and between the sequence KY522837 and that of *M. holsaticum*.

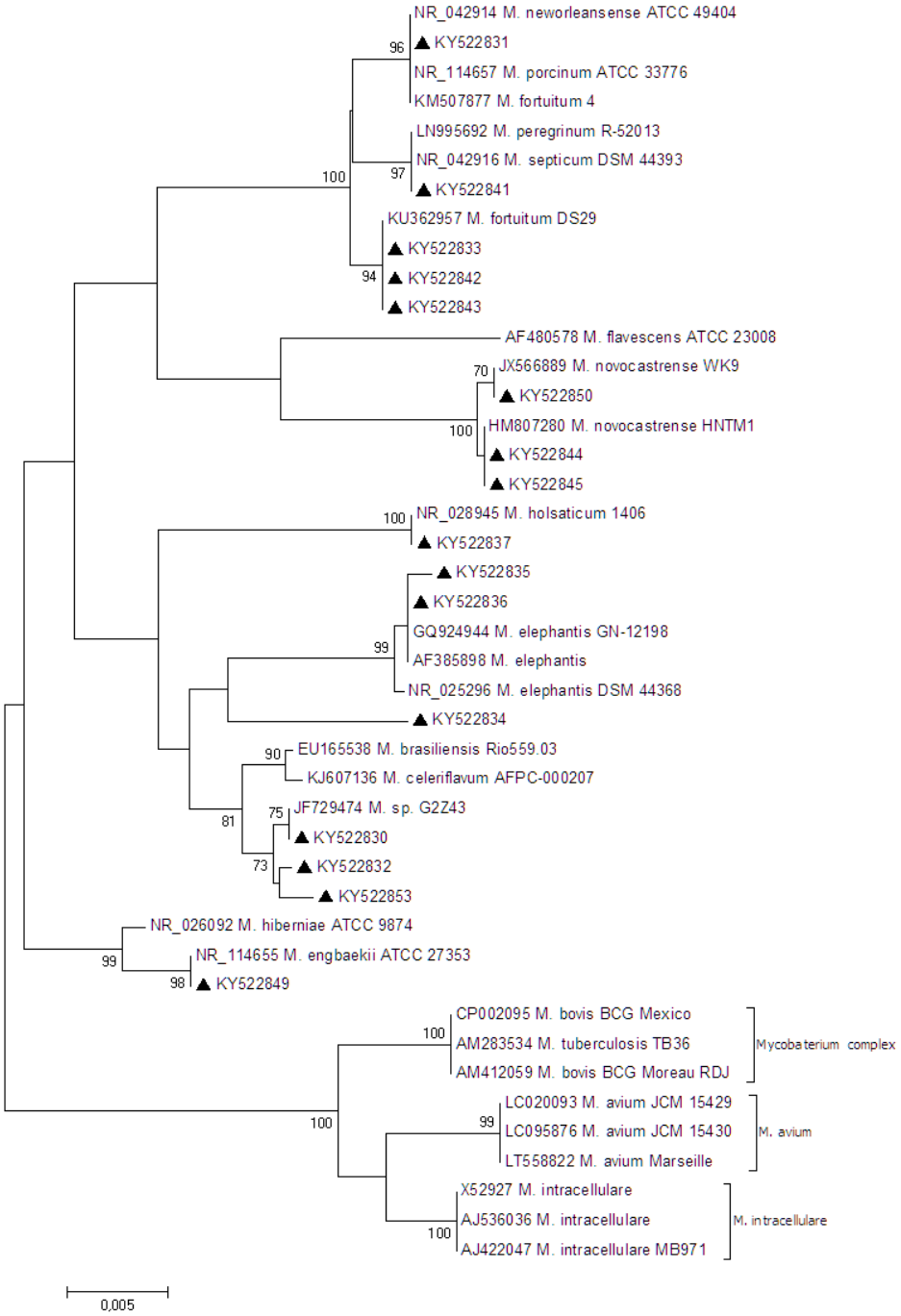

Figure 1 – Reconstructed phylogenetic tree generated using the neighbor-joining method based on the maximum composite likelihood substitution model for a 881-nucleotide fragment of the mycobacterial 16S rRNA gene. Sequences are identified according to their respective accession numbers; those determined in this study were indicated with a triangle. The numbers above each node represent the bootstrap values, but only those greater than 70% were presented. The scale represents the number of substitutions/site

Although the isolation and/or PCR detection of *M. bovis* have been reported in raw milk or fresh cheese (LEITE et al., 2003, VIALTA et al., 2003, HARRIS et al., 2007; FRANCO et al., 2013; ZARDEN et al., 2013, CEZAR et al., 2016), we were unable to detect this species in the samples analyzed in this study.

Variable results regarding the ability to detect *M. bovis* in examined samples may be attributable to a range of factors that influence the sensitivity and specificity of the different protocols used. These factors include the low prevalence of the disease in the Brazilian bovine herd (BAHIENSE et al., 2016; BARBIERI et al., 2016; DIAS et al., 2016; GALVIS et al., 2016; GUEDES et al., 2016; LIMA et al., 2016; NÉSPOLI et al., 2016; QUEIROZ et al., 2016; RIBEIRO et al., 2016; ROCHA et al., 2016; SILVA et al., 2016; VELOSO et al., 2016; VENDRAME et al., 2016), the dilution effect of contaminated milk mixed with uncontaminated milk, the low multiplication probability of the initial microbial population in milk as a function of storage time and temperature, the reduction in contaminant load during production and curing (STARIKOFF et al., 2016), and the lack of a validated research method for *M. bovis* in food products that enables the detection of low levels of mycobacteria in high levels of other microorganisms. The presence of this accompanying microbiota calls for sample decontamination, which, by causing injury in bacterial cells, might also have hindered the detection of *M. bovis* (CORNER et al., 1995).

The isolation of non-tuberculous mycobacteria (Figure 1) corroborates the findings of researchers who have detected this group in both raw and pasteurized milk and cheese samples (LEITE et al., 2003; HARRIS et al., 2007; FRANCO et al., 2013; SGARIONI et al., 2014), indicating that the isolation of non-tuberculous mycobacteria from milk and dairy products is common.

Non-tuberculous mycobacteria have been recognized as pathogens and are among the causes of human infections (WU et al., 2009; VARGHESE et al., 2013). These bacteria account for 0.5% to 35% of all mycobacterial infections in humans (KONEMAN et al., 2001) and are present in 50% of HIV-positive patients (FALKINHAM, 1996), indicating the importance of these agents in immunosuppressed individuals (ARASTÉH et al., 2000). To date, no epidemiological evidence was found for the classification of non-tuberculous mycobacteria as disease agents transmitted by food; the oral route has been indicated as the gateway for these agents (HORSBURGH JUNIOR; SELIK, 1989; REDDY, 1998; YODER et al., 1999; TORTOLI, 2004), thereby suggesting food as a possible route of infection transmission.

Although it was not possible to identify the mycobacterial species isolated in this study (Figure 1), the constructed phylogenetic tree (Figure 1) shows that the isolates obtained were grouped near *M. novocastrense* (3 samples), *M. holsaticum* (1 sample), and *M. elephantis* (2 samples). The latter species was initially isolated from a lung abscess of an elephant that died of chronic respiratory disease (SHOJAEI et al., 2000). Subsequently, this species has been isolated from 11 Canadian patients with respiratory diseases, although its clinical relevance remains undetermined (TURENNE et al., 2002). *M. holsaticum* was first described in 2002 from clinical specimens (sputum, urine, gastric fluid) of patients living in different regions of Germany (RICHTER et al., 2002), whereas *M. novocastrense* was initially isolated in the United Kingdom, in 1997, from the skin lesion of a child (SHOJAEI et al., 1997), and it has more recently been isolated in water sources (plant irrigation water, public parks, and a hospital supply system) in Iran (DIBAJ et al., 2014). *M. novocastrense* has also been isolated from two samples of raw milk taken from collective milk tanks in São Paulo, Brazil (FRANCO et al., 2013). To date, however, no evidence of the transmission of these mycobacteria via food was found.

Considering that non-tuberculous mycobacteria are ubiquitous and therefore inhabit a wide variety of environments (PRIMM et al., 2004), the contamination of the cheese analyzed in this study might have occurred at any stage of the production process: milking, the cheese processing steps (in which large amounts of water are required and hygiene conditions are probably not rigidly controlled), and in product retail sites where these cheeses remain exposed, without packaging. The presence of these mycobacteria reinforces the need for quality control measures, such as hazard analysis and critical control point programs. In addition, the findings of this study suggest the control of the product at the point of sale.

Our findings also highlight the need for a specific determination procedure that favors the isolation of *M. bovis* from food samples characterized by high loads of accompanying microorganisms. In addition, the study underlines the necessity for the scientific community to be aware of potential health risks associated with non-tuberculous mycobacteria, particularly among the immunosuppressed individuals.

Conclusion

The isolation of non-tuberculous mycobacteria from more than 10% of the Minas cheese samples we examined indicates the need to improve hygiene conditions along the food production chain. Although these mycobacteria cannot currently be claimed to have pathogenic potential when transmitted via food, their ability to cause disease should not be ignored, particularly among the immunosuppressed population.

Conflict of interest

The authors declare no conflicts of interest.

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