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Full Length Article

Detection of *Mycobacterium bovis* in artisanal cheese in the state of Pernambuco, Brazil

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ABSTRACT

Objective/background: The present study was aimed at detecting *Mycobacterium bovis* in artisanal cheese using real-time quantitative polymerase chain reaction.

Methods: One hundred seven cheese samples (250 g) were purchased in 107 commercial establishments including neighborhood grocery stores, bakeries, and open-air markets from 19 municipalities of the state of Pernambuco, Brazil. Ten grams of each cheese sample were macerated with sterile saline solution in a sterile bag and DNA was extracted from 20 mg of the macerated material using the Wizard SV Genomic DNA Purification System. The quantitative polymerase chain reaction amplified a fragment corresponding to the region of difference 4 of *M. bovis*.

Results: Of the 107 samples analyzed, three (2.8%) were positive for *M. bovis* and their identities were confirmed by sequencing. This is perhaps the first report of the presence of *M. bovis* in artisanal cheese in the state of Pernambuco, Brazil.

Conclusion: The results of the present study highlight the need for improving sanitary measures during the production of artisanal cheese to prevent zoonotic tuberculosis in humans, resulting from the consumption of food contaminated with *M. bovis*.

Introduction

Artisanal cheeses are produced with raw milk in small farms around the world [1]. The production of artisanal cheese often does not comply with the hygienic–sanitary requirements demanded by official agencies, especially regarding to the possibility of being contaminated with pathogens due to the

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use of raw milk from unhealthy dairy cows and lack of hygiene during processing [2].

Mycobacterium bovis is the etiological agent of bovine tuberculosis and can cause human tuberculosis, in which clinical symptoms are indistinguishable from those caused by *Mycobacterium tuberculosis*. Consumption of milk and its derived products contaminated with *M. bovis* represent the main infection route of zoonotic tuberculosis for humans [3,4].

The presence of the bacillus in cheese and its association with human tuberculosis has already been reported [5–7]. In Brazil, studies have demonstrated the presence of *M. bovis* in raw cattle milk [8,9].

Considering the importance of artisanal cheese in Brazil and its participation in the transmission chain of zoonotic tuberculosis to humans, the aim of the present study was to detect *M. bovis* in artisanal cheeses commercialized in the state of Pernambuco, Brazil, using molecular techniques.

Material and methods

Sampling

One hundred and seven samples of artisanal cheese "*coalho*" type were purchased in 107 commercial establishments including neighborhood grocery stores, bakeries, and open-air markets located in 19 municipalities of the Garanhuns microregion, state of Pernambuco, Brazil, as follows: Angelim (six samples), Bom Conselho (six samples), Brejão (six samples), Caetés (six samples), Calçado (seven samples), Canhotinho (six samples), Correntes (six samples), Garanhuns (six samples), Iati (four samples), Jucati (six samples), Jupi (eight samples), Jurema (five samples), Lagoa do Ouro (six samples), Lajedo (six samples), Palmerina (three samples) Paranatama (five samples), Saloá (six samples), São João (six samples), and Terezinha (three samples).

The cheese samples were sent to the Garanhuns Laboratories Center, located in the Garanhuns Academic Unit of the Federal Rural University of Pernambuco in an isothermal box containing reusable ice.

DNA extraction

The cheese samples (250 g) were partitioned and 10 g of cheese were macerated with 20 mL of 0.9% sterile saline solution in a sterile bag. DNA extractions were performed with 20 mg of the macerated material using the Wizard SV Genomic DNA Purification System (Promega. Promega Corporation, USA, 2012) following the manufacturer's instructions.

Positive control

The *M. bovis* American Type Culture Collection (ATCC) 19274 strain was provided by the Oswaldo Cruz Foundation (Fundação Osvaldo Cruz, Rio de Janeiro, Brazil) and it was used for the construction of a plasmid harboring the target sequence, which was the positive control in the molecular tests. The genomic DNA of *M. bovis* ATCC 19274 strain was extracted and the fragment corresponding to the region of difference 4 (RD4) was amplified with the specific primers reported by Sales et al. [10]. The target fragment was cloned using *Escherichia coli* XL1 blue strain and TA cloning kit (Invitrogen. Invitrogen Corporation, Califórnia, 2006) according to the manufacturer's instructions.

Real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed using the same primer set used for the amplification of the RD4 fragment of the *M. bovis* ATCC 19274 strain in the presence of TaqMan Universal PCR Master Mix (Applied Biosystems, USA, 2010) and DNA sample. The reaction solution (25 μL) consisted of 2.0 μL of DNA and 12.5 μL of the master mix with 1.0 μL of each primer (5 pmol), 0.5 μL of probe (5 pmol), and 8-μL water. The amplification conditions were 95 °C for 15 min (denaturation) followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s. The qPCR was performed in an ABI 7500 Real-Time PCR (Applied Biosystems, USA, 2010) system set for absolute quantification. In all PCR runs, standard curves were obtained using plasmid DNA encompassing the mycobacteria RD4 sequence as positive control. The positive control was prepared in triplicate by serial dilution of 10× plasmid DNA from 200 ng (quantification cycle = 11.8) to 0.0002 ng (quantification cycle = 32.2). The standard curve slope was −3.40 and $R = 0.999$ with 97% efficiency.

Sequencing

The commercial kit ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction v3.1 (Applied Biosystems, USA, 2009) was used for DNA sequencing following the manufacturer's recommendations. The RD4 fragments were sequenced using the Sanger method and the reaction products were analyzed in the ABI 3500xL Genetic Analyzer (Applied Biosystems, USA, 2009).

All the sequences obtained in the present study were compared with the RD4 fragment of the reference genome (88 bp; GenBank Access number BX248339.1) using the software Blast-N (http://www.ncbi.nlm.nih.gov) and MEGA6 [11].

Results and discussion

This is the first report on the occurrence of *M. bovis* in artisanal cheese commercialized in the Garanhuns microregion, a dairy region of the state of Pernambuco, northeastern Brazil.

Of the 107 samples analyzed in the present study, three (2.8%) were positive for *M. bovis* and their identities were confirmed by sequencing. The positive samples were from two neighborhood grocery stores of the municipality of Correntes (one out of six) and Lagoa do Ouro (one out of six), and an open-air market located in Bom Conselho (one out of six; Table 1).

Few studies in Brazil have evaluated the presence of *M. bovis* in cheese. In the present study, *M. bovis* DNA was present in 2.8% of the samples analyzed, which is lower than the 10% positivity found in samples of the same type of cheese collected in the state of Piauí, northeastern Brazil [12]. Our study and the one of Silva et al. [12] shows the risk of human infection by *M. bovis* through the consumption of cheese, yogurt, and milk without heat treatment, as 41% of milk is illegally

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