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Detection of mycobacteria in coalho cheese sold in Northeastern Brazil

Detecção de micobactérias em queijo de coalho comercializado no Nordeste do Brasil

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Highlights

Two samples (4%) showed mycobacterial growth.
PCR was effective in detecting *hsp65* of *Mycobacterium* spp. in cheese.
Phylogenetic analysis showed similarity with *M. lehmannii* and *M. rutilum*.

Abstract

The aim of the present study was to detect and identify *Mycobaterium* spp. in 50 samples of coalho cheese sold in the Northeast region of Brazil. Of the 50 analyzed samples, 35 were produced by the artisanal process, using raw milk, and 15 originated from industrialized establishments that pasteurize milk. Conventional and real-time nested PCR for the *rv2807* gene of the *M. tuberculosis* complex was performed directly from the 50 analyzed samples. Samples of coalho cheese were grown simultaneously in Stonebrink medium, and conventional PCR was performed from the bacterial isolates with primers that flank differentiation region 4 (DR4), specific to *M. bovis*, mb400F. Bacterial isolates negative by PCR for RD4 were subjected to PCR for *hsp65* of *Mycobacterium* spp., with subsequent DNA sequencing. The cultures were negative for the *M. tuberculosis* complex, but two samples (4%) from the artisanal process (with raw milk) exhibited identity with *hsp65* of *Mycobacterium lehmanii* (Sequence ID: KY933786.1, identities: 312/363 [86%]); and *Mycobacterium rutilum* (sequence ID: LT629971.1, identities: 331/371 [89%]), showing to be indicative environmental contamination. Non-tuberculous mycobacteria are emergent and ubiquitous microorganisms; therefore, they deserve greater attention in the cheese production chain, both in terms of Good Agricultural Practices (GAP) and food Good Manufacturing Practices (GMP).

Key words: Coalho cheese. *Mycobacterium lehmanii*. *Mycobacterium* ssp. *Mycobacterium rutilum*. Nested-PCR.

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Resumo

O presente estudo teve como objetivo detectar e identificar *Mycobaterium* spp. em 50 amostras de queijo de coalho comercializados na região Nordeste do Brasil. Das 50 amostras analisadas, 35 foram produzidas pelo processo artesanal, com leite cru e 15 provenientes de estabelecimentos industrializados que realizam a pasteurização do leite. Nested-PCR convencional e em tempo real para o gene *rv2807* do Complexo *M. tuberculosis* foi realizada diretamente das cinquenta amostras analisadas. Paralelamente, as amostras de queijo de coalho foram cultivadas em meio de Stonebrink e dos isolados bacterianos foi realizada PCR convencional com iniciadores que flanqueiam a região de diferenciação 4 (RD4), específica de *M. bovis*, mb400F. Os isolados bacterianos negativos na PCR para RD4 foram submetidos à PCR para hsp65 de *Mycobacterium* spp., com posterior sequenciamento do DNA. As culturas mostraram-se negativas para o Complexo *M. tuberculosis*, porém duas amostras oriundas do processo artesanal (com leite cru) (4%) apresentaram identidade com *hsp65* de *Mycobacterium lehmanii* (ID da sequência: KY933786.1, identidades: 312/363 [86%]); e *Mycobacterium rutilum* (ID da sequência: LT629971.1, identidades: 331/371 [89%]), apontando-se como indicativos de contaminação ambiental. As micobactérias-não-tuberculosas (MNT) são microrganismos emergentes e de natureza ubíqua. Devido a essas características merecem maior atenção na cadeia produtiva de queijos, tanto nas boas práticas agropecuárias (BPAs) quanto nas boas práticas de fabricação de alimentos (BPFs).
Palavras-chave: Queijo de coalho. *Mycobacterium lehmanii*. *Mycobacterium* ssp. *Mycobacterium rutilum.* Nested-PCR.

Introduction

At present, the genus *Mycobacterium* comprises about 170 species, including several important human pathogens and numerous non-tuberculous species. Although some are notable pathogens, such as *M. tuberculosis*, *M. bovis* and *M. leprae*, most are environmental organisms that act majorly as opportunistic infectious agents, usually in chronic patients (Tortoli, 2014).

Consumption of milk and its derivatives contaminated with *M. bovis* constitutes the main route of infection of zoonotic tuberculosis for humans. Despite this, Nunes-Costa, Alarico, Pretti Dalcolmo, Correia-Neves and Empadinhas, (2016) emphasized the impact of non-tuberculous mycobacteria (NTM) on the morbidity and mortality of patients with malignant, autoimmune and immunosuppressed diseases, which have considerably increased in recent decades.

The consumption of artisanal coalho cheeses from street markets represents a risk to human health due to the possibility of transmission of potentially pathogenic NTM (Sarti et al., 2018). Some artisanal cheeses are typically produced with milk that does not undergo thermal treatment on small farms. In addition, artisanal production does not always meet good hygiene practices, which makes the consumption of this product a potential route of transmission of infectious agents. It is noteworthy that artisanal coalho cheese is a popular dairy product widely consumed in the Northeast region of Brazil that is produced from raw milk by adding coalho.

In view of the importance of coalho cheese consumption in northeastern Brazil and its possible participation in the epidemiological

chain of foodborne diseases, this study was conducted to detect *Mycobacterium* spp. in this cheese type (artisanal and industrialized), sold in the *sertão* (hinterlands) of Rio Grande do Norte, northeast Brazil, through bacteriological cultivation and molecular detection.

Fifty samples of coalho cheese were collected in the retail market of the municipality of Caicó, which is located in the Central Mesoregion of the state of Rio Grande do Norte, northeast Brazil. Livestock and the dairy sector in this region are of fundamental importance for the agricultural and regional socio-spatial organization. In this region, cheese makers amount to 314 units that use 42% of all milk production for the manufacture of various types of products, such as butter, coalho cheese and ricotta cheese. Their annual production is 315 t and they serve the consumer market in several states of the country (Agência de Desenvolvimento Sustentável de Seridó [ADESE], 2011).

Of the total of 50 samples of coalho cheese analyzed, 35 were produced by the artisanal process, using raw milk, and 15 originated from industrialized establishments that pasteurize milk. The samples were packed in plastic bags, frozen at -20 ºC and later transported in cooler boxes to the laboratory for further processing.

Two-gram aliquots of each cheese sample were macerated and homogenized. For the extraction of genetic material, the DNeasy Mericon Food kit (Qiagen, Hilden, Germany) was used, following the manufacturer's recommendations. The concentration of extracted DNA was determined using Qubit (Invitrogen, Waltham, USA), and its integrity in 1% agarose gel.

The nested-PCR for the *rv2807* gene of the *M. tuberculosis* complex (MTC) was performed according to the protocol described by Araújo et al. (2014). Two sets of primers were designed, namely, external primers for amplification by conventional PCR, internal primers and probe for amplification by real-time PCR. The primer and probe sequences are shown in Table 1.

Table 1
Primers and probe used in Nested-PCR to detect the *rv2807* gene of the *Mycobacterium tuberculosis* complex in coalho cheeses

Target site	DNA sequences (5′ → 3′)
rv2807	External F: GGCGGTGGCGGAGTTGAAGGCGATG
	External R: GCCGCGAGCGAGTCTGGGCGATGTC
	Probe: 6FAM CATCCACACCTGTTCG MGBNFQ
	Internal F: CATTGCTGCGTAATTCGATCA
	Internal R: GACCTTGGGGCGCTCAT

During the first stage, conventional PCR was performed using 10 pmol of each primer, 10 mM dNTPs (Invitrogen, Waltham, USA), 2.5 µL of 10 × buffer (Sigma-Aldrich, St. Louis, USA), 1.25 U of Taq DNA polymerase (Sigma-Aldrich, St. Louis, USA) and 100 ng of DNAe, in a final reaction volume of 25 µL. Amplification conditions were 95 ºC for 4 min, 35 cycles of 95 ºC for 90 s, 65 ºC for 30 s, 72 ºC for 45 s and a final extension at 72 ºC for 3 min. Real-time PCR amplifications were performed in the second stage, using 5 pmol of each primer (Applied Biosystems, Foster City, USA), 5 pmol of TaqMan probe (Applied Biosystems, USA), 6.25 µL of Master Mix PCR TaqMan (Applied Biosystems, Foster City, USA) and 3 µL of the conventional PCR reaction, performed during the first stage, in a final reaction volume of 12.5 µL. The conditions for real-time PCR amplification were as follows: 95 ºC for 10 min for initial denaturation, 35 cycles of denaturation at 95 ºC for 15 s and annealing/extension at 62 ºC for 30 s. All reactions were performed in a StepOne Plus thermocycler (Applied Biosystems, Foster City, USA), in duplicate, using template DNA from *M. bovis* AN5 as positive-reaction controls.

In parallel, 5 g of each coalho cheese sample were macerated with 5 mL of sterile 0.9% NaCl solution (saline). Next, 40 mL of sterile 0.9% saline were added to the solution, which was followed by homogenization. From this mixture, 5 mL were separated and centrifuged for 15 min at 1,000 x g. The intermediate phase was discarded and 5 mL of sterile type-I water were added to the sediment, followed by shaking. Then, 5 mL of the suspension were transferred to a clean tube and 3 mL of sodium lauryl sulfate were added, followed by shaking; the tube containing the material was incubated at 37° C for 20 min and shaken at every 5 min. Subsequently, three drops of phenol red, five drops of 1N HCl and nine drops of 0.4% neutralizing solution were added. Eight drops were then deposited in a tube with Stonebrink medium, in duplicate, which were incubated at 37 ºC without the use of CO_2, for up to 90 days, and checked weekly for the growth of mycobacteria (Thoen, Himes, Jarnagin, & Harrington, 1979).

The DNA extraction protocol for bacterial isolates obtained in Stonebrink medium was based on the thermolysis method, following De Medici et al. (2003). The DNA concentration was checked using a Qubit apparatus (Invitrogen, Waltham, USA) and its integrity by agarose gel with GelRed staining (Sigma-Aldrich, St. Louis, USA).

Once the DNA of the bacterial isolates was obtained, PCR was performed with primers that flank differentiation region 4 (RD4), specific to *M. bovis*, namely, mb400F, AACGCGACGACCTCATATTC and mb400R: AAGGCGAACAGATTCAGCAT, which amplify a 400-bp fragment. The reaction was carried out using 10 pmol of each primer, 10 mM dNTPs (Invitrogen, Waltham, USA), 2.5 µL of 10 × buffer (Sigma-Aldrich, St. Louis, USA), 1.25 U of Taq DNA polymerase (Sigma-Aldrich, St. Louis, USA) and 100 ng of DNA, in a final reaction volume of 25 µL. The PCR amplification conditions were 94 ºC for 5 min and 35 cycles at 94 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 35 s, with a final extension at 72 ºC for 3 min.

Bacterial isolates negative by PCR for RD4 were subjected to PCR for *hsp65* of *Mycobacterium* spp., for subsequent DNA sequencing. For this, primers Tb11: ACCAACGATGGTGTGTCCAT and Tb12:

CTTGTCGAACCGCATACCCT were used for *Mycobacterium* spp., following Telenti et al. (1993). The reaction was performed using 10 pmoL of each primer, 10 mM dNTPs (Invitrogen, Waltham, USA), 2.5 µL of 10 × buffer (Sigma-Aldrich, St. Louis, USA), 1.25 U of Taq DNA polymerase (Sigma-Aldrich, St. Louis, USA) and 100 ng of DNA, in a reaction volume of 25 µL. The conditions for PCR amplification were as follows: 45 cycles of denaturation for 60 s at 94 ºC, annealing for 60 s at 60 ºC and extension at 72 ºC, followed by a 10-min extension at 72 ºC.

Subsequently, the PCR products were purified using ExoSAP (ThermoFischer, Waltham, USA) and sequenced in duplicate using the BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, Foster City, USA). The resulting consensus DNA sequences were subjected to identity search using Blastn (NCBI).

The coalho cheese samples were negative by nested-PCR for MTC (*rv2807* gene). However, two samples (4%) from the artisanal process (with raw milk) showed identity with *hsp65* of *Mycobacterium lehmanii* (sequence ID: KY933786.1, identities: 312/363 [86%]); and *Mycobacterium rutilum* (sequence ID: LT629971.1, identities: 331/371 [89%]).

In Brazil, research investigating the presence of mycobacteria in coalho cheese is still incipient. In a study conducted by Rocha (2013) on coalho cheeses produced in the state of Piauí, molecular evidence of *M. bovis* was found in 10% of the analyzed material. This generated an alert to the authorities about the need for further research aiming at the search of this pathogen in humans, animals and food, as well as studies aiming at uniformity in the artisanal cheese production process, to ensure the safety of this product.

Non-tuberculous mycobacteria are usually found in water, soil and dust. Until not long ago, NTM were not associated with pathogens in humans, but, recently, with the advent of molecular diagnosis, they have been constantly mentioned in medical research articles (Falkinham, 2013). Recent molecular biology studies corroborate this information, presenting new species, e.g. Nouioui et al. (2017), who proposed the creation of two new species of fast-growing mycobacteria: *M. lehmannii* sp. nov. and *M. neumannii* sp. nov., respectively.

Although most studies seek to detect *M. bovis* and M*. avium* subsp. *paratuberculosis* (MAP) in cheeses produced with raw milk, given the zoonotic potential of these agents, recent studies have aspired to detect other mycobacteria. This can be seen in the study of Sarti et al. (2018), who analyzed coalho cheeses sold in the state of Paraíba, Brazil, and detected contamination of 5% (n = 100) with NTM (*M. fortuitum*, *M. novocastrense*).

Currently, NTM are emerging microorganisms, and due to their ubiquitous nature, they deserve special attention, especially in the production of dairy products. The identification of NTM in coalho cheeses makes greater sanitary control of this product necessary in producing regions in order to ensure its quality.

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Conflict of interests

The authors declare no conflict of interest.

Authors' contributions

All authors contributed equally to the conceptualization and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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