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Isolation and identification of *Mycobacterium bovis* in milk from cows in northeastern Brazil

Isolamento e identificação de *Mycobacterium bovis* em leite de vacas no Nordeste do Brasil

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— NOTE —

ABSTRACT

*Milk samples from 16 cows that tested positive on the tuberculin test in the state of Paraíba, northeastern Brazil, were used for mycobacteria isolation and identification. Mycobacteria were isolated from five (31.25%) of the 16 milk samples; three samples were classified as **M. bovis**, and two as belonging to the **Mycobacterium** genus. This is probably the first study of isolation and identification of **M. bovis** in milk from cows in Northeastern Brazil, which suggests that humans are at risk of contamination by ingestion.*

Key words: ***Mycobacterium bovis**, isolation, milk, PCR, cattle.*

RESUMO

*Amostras de leite de 16 vacas positivas no teste da tuberculinização no Estado da Paraíba, Nordeste do Brasil, foram utilizadas para isolamento e identificação de micobactérias. Foram isoladas micobactérias em cinco (31,25%) das 16 amostras de leite: três amostras foram classificadas como **M. bovis** e duas como pertencentes ao gênero **Mycobacterium**. De acordo com nosso conhecimento, este é o primeiro estudo de isolamento e identificação de **M. bovis** em leite de vacas no Nordeste do Brasil, o que sugere que os seres humanos estão em risco de contaminação por ingestão.*

Palavras-chave: ***Mycobacterium bovis**, isolamento, leite, PCR, bovinos.*

Bovine tuberculosis is a chronic, zoonotic disease caused by ***Mycobacterium bovis***, which belongs to the ***Mycobacterium tuberculosis*** complex. The disease is responsible for economic losses due to reduced reproductive efficiency, decreased milk production and decreased weight gain, carcass condemnation, and restrictions on international trade of animals and animal products (BRASIL, 2006).

While most cases of tuberculosis in humans are caused by ***M. tuberculosis***, it is estimated that about 3.1% of the cases of human tuberculosis in the world are caused by ***M. bovis*** (EL-SAYED et al., 2016). However, infection in humans is generally not confirmed by isolation and identification of the agent, making it impossible to identify the possible source of infection. In addition, the human diseases caused by ***M. tuberculosis*** and ***M. bovis*** are indistinguishable using clinical, radiological and pathological methods (ROCHA et al., 2011). Transmission of ***M. bovis*** to humans occurs through consumption of raw milk and dairy products, or through contact with secretions from fistulated abscesses.

In the state of Paraíba, official data indicated the occurrence of 1,015 cases of human tuberculosis in 2014 (BRASIL, 2015). In areas where human and bovine tuberculosis coexist, the differentiation between ***M. bovis*** and ***M. tuberculosis*** is important for monitoring the spread of ***M. bovis*** among cattle and from cattle to humans. Thus, the objective of this study was to isolate and identify ***M. bovis*** in milk from cows with a positive diagnosis identified using Single

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Intradermal Comparative Cervical Tuberculin (SI CCT) test in the state of Paraíba, Northeastern Brazil.

SICCT test was performed by veterinarians of the Official Veterinary Service of the Paraíba State following the strategies of the National Program for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT); an animal was considered positive to the SICCT test when the 72-hour reaction to bovine tuberculin (Bov) was greater than the reaction to avian tuberculin (Av) by more than 4mm (BRASIL, 2006). All SICCT-positive animals were euthanized according to PNCEBT technical regulation.

Milk samples from 16 mixed-breed cows with average age of 6.4 years that tested positive on the SICCT test from seven different properties located in the municipalities of Cacimba de Areia, Patos, Piancó and São Mamede were used (Figure 1). Animals did not show any clinical signs of tuberculosis, chronic mastitis or local lymphadenopathy. Properties were labeled A (Coordinates: 07º13'28.9"S 37º56'21.7"W), B (07º08'41.7"S 37º07'16.7"W), C (06º56'41.7"S 37º10'22.6"W), D (06º58'37.3"S 37º16'38.7"W), E (06º57'53.5"S 37º20'32.2"W), F (06º58'36.0"S 37º17'21.9"W) and G (07º00'24.3"S 37º16'15.6"W). Milk was collected directly from the teats of the animals before milking, following all methods of antisepsis. An average of 15mL of milk per animal was collected, utilizing sterile and previously identified vials. These were sent to the laboratory in isothermal boxes with ice.

For the isolation of ***Mycobacterium*** spp., a 5mL aliquot of milk was used, centrifuged at 3000rpm for 20 minutes. In this way, two phases of milk were obtained, fat and sediment, which were packaged in different vials and both decontaminated using the Petroff method (KANTOR, 1979), inoculated in duplicate in Stonebrink and Lowenstein-Jensen media, and incubated at 37°C for 90 days. Colonies suggestive of mycobacteria were stained using the Ziehl Neelsen method; DNA was extracted using thermolysis (MAZARS et al., 2001). Samples identified as Acid Fast Bacilli (AFB) in Ziehl Neelsen were subjected to molecular identification.

Identification of ***Mycobacterium*** species and differentiation of the ***M. tuberculosis*** complex, ***M. avium*** complex, ***M. intracellurare*** complex, and other ***Mycobacterium*** spp. was done

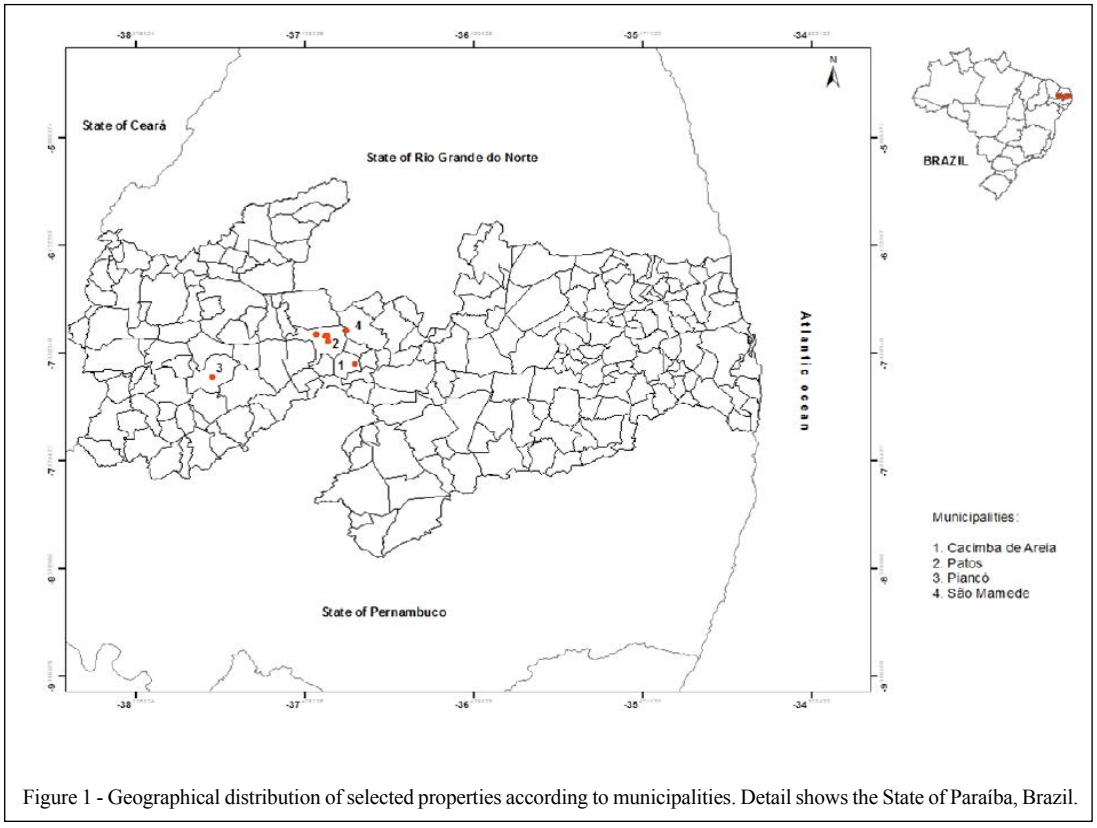

Figure 1 - Geographical distribution of selected properties according to municipalities. Detail shows the State of Paraíba, Brazil.

using the TB Multiplex PCR (WILTON & COUSINS, 1992). For this, the primers used were: MYCGEN-F (G1) (5'-AGAGTTTGATCCTGGCTCAG-3') and MYCGEN-R (G2) (5'-TGCACACAGGCCACAAGGGA-3') – associated with genus; TB-1F (5'-GAACAATCCGGAGTTGACAA-3') and TB-1R (5'-AGCACGCTGTCAATCATGTA-3') - related to the ***M. tuberculosis*** complex; MYCAV-R (5'-ACCAGAAGACATGCGTCTTG-3') – related to ***M. avium***; and MYCINT-F (5'CCTTTAGGCGCATGTCTTTA-3') – related to ***M. intracellulare***. Positive controls used were the ***M. bovis*** strain AN5 and ***M. tuberculosis*** strain H37Rv for the ***M. tuberculosis*** complex. The 50µL reactions were performed containing reaction buffer dNTP (1.25mM each), 20pmol of each oligonucleotide, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 10pmol μL^{-1} of primers, 1.25 units of TAQ polymerase (1.0µL) and 5µL of the DNA under study. Amplification cycles used were the following: once at 94°C for 10 minutes, 61°C for 2 minutes, and 72°C for 3 minutes; 33 times at 94°C for 30 seconds, 61°C for 2 minutes, and 72°C for 3 minutes; once at 94°C for 30 seconds, 61°C for 2 minutes, and 72°C for 10 minutes. Samples containing an amplification of 1030bp were identified as belonging to the ***Mycobacterium*** genus, and samples with 372bp fragments were considered members of the ***M. tuberculosis*** complex (WILTON & COUSINS, 1992).

All DNA samples with consistent amplification for ***M. tuberculosis*** complex using the TB Multiplex PCR were amplified with the following primers: RD4 (RD4-1 5'-ATG TGC GAG CTG AGC GAT G-3'; RD4-2 5'-TGT ACT ATG CTG ACC CAT GCG-3'; and RD4-3 5'-AAA GGA GCA CCA TCG TCC AC-3') for identification *of* ***M. bovis*** (WARREN et al., 2006). 25µL reactions were conducted containing the reaction buffer dNTPs (1.25mM each), 20pmol of each oligonucleotide, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, primers, 1.25 units of TAQ polymerase, and 5µL of the genomic DNA being studied. Positive controls used were the ***M. bovis*** AN5 strain and ***M. tuberculosis*** strain H37Rv. PCR cycles used were: once at 95°C for 15 minutes, 45 times at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 min, and once at 72°C for 10 minutes. Sizes of the PCR products indicate that the region studied is absent or present in the respective members of the ***M. tuberculosis*** complex. Thus, the 268bp fragment resulting from amplification is consistent with the RD4 absent in the sample, characteristic of the ***M. bovis*** species.

Mycobacteria were isolated from five (31.25%) of the 16 milk samples; three samples were classified as ***M. bovis,*** and two as belonging to the ***Mycobacterium*** genus. This is the first study of isolation and identification of ***M. bovis*** in milk from cows in Northeastern Brazil. Several studies have been conducted in other states in order to isolate and identify mycobacteria in milk from cows. In São Paulo, PARDO et al. (2001) isolated ***M. bovis*** in one sample of raw milk; LEITE et al. (2003), using milk samples from the states of São Paulo, Paraná, Santa Catarina, Goiás and Pará, isolated ***M. bovis*** in one sample of raw milk. FRANCO et al. (2013), in the southeast region of São Paulo, isolated ***M. bovis*** from one milk sample from an individual cooling tank.

Milk and its derivatives, when contaminated and unpasteurized, present a significant risk of tuberculosis transmission to humans. It is estimated that around 50% or more of milk produced in Brazil is unpasteurized (LEITE et al., 2003), attributing serious risks of transmission of various diseases to humans, including tuberculosis caused by ***M. bovis***. It is worth highlighting that it is common in northeastern Brazil to consume raw milk.

An important aspect was the isolation and identification of ***Mycobacterium*** spp. in two samples, probably nontuberculous environmental mycobacteria (NTM), which are present in the environment and can be transmitted to animals and humans (FRANCO et al., 2013) through inhalation or ingestion, resulting in permanent or temporary colonization of the respiratory and digestive tracts (PRIMM et al., 2004). The increasing number of individuals infected with HIV predisposes an increase in the number of cases of emerging and re-emerging diseases, especially those caused by opportunistic agents, such as ***Mycobacterium*** spp. In this context, non-pasteurized milk infected with mycobacteria poses a serious risk to individuals with HIV (FRANCO et al., 2013).

The presence of ***M. bovis*** in milk from cows in the state of Paraíba in Northeastern Brazil suggests that humans are at risk of contamination by ingestion. This reinforces the need for optimization of milk and dairy products quality programs, enhancing sanitary inspections of these products, and conducting studies for isolating and identifying mycobacteria in milk from other northeastern states.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the institutional Committee for Ethics in the Use of Animals (Universidade Federal de Campina Grande – UFCG) (protocol 25-2012).

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