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Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk from the state of Pernambuco, Brazil

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ABSTRACT

The aim of this study was to detect the IS900 region of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine milk samples using real-time polymerase chain reaction (qPCR) and conventional PCR, and to study the agreement between these tests. A total of 121 bovine milk samples were collected from herds considered positive for MAP, from the State of Pernambuco, Brazil. MAP DNA was detected in 20 samples (16.5%) using conventional PCR and in 34 samples (28.1%) using qPCR. MAP DNA was detected in all of the 6 animal farms studied. Moderate agreement was found between qPCR and conventional PCR results, where the sensitivity and specificity of conventional PCR in relation to qPCR were 50% and 96.6%, respectively. Thus, the IS900 region of MAP was found in bovine milk samples from the State of Pernambuco. To the best of our knowledge, this is the first report of MAP DNA found in bovine milk in Northeast Brazil. We also demonstrated the qPCR technique is more sensitive than conventional PCR with respect to detection of MAP in milk samples.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a gram-positive, slow-growing bacillus of the family *Mycobacteriaceae* that possesses a cell wall rich in lipids, characteristic of this family. This microorganism is an intracellular pathogen, responsible for Johne's disease or paratuberculosis.[1,2]

Paratuberculosis has been previously investigated in several studies in Brazil.[3] Its occurrence in Pernambuco has been described in dairy cattle based on clinical signs, histopathology, and results from enzyme-linked immune sorbent assay (ELISA) serology (32.3% positive samples), isolation (50% positive samples), and polymerase chain reaction (PCR) studies.[4]

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Additionally, a prevalence rate of 2.7% (11/408) has been reported in the micro-region of Garanhuns, with 47.4% (9/19) outbreaks.[5]

The most common methods of MAP diagnosis in infected animals include isolation of bacteria from feces using selective culture media and antibody detection techniques such as ELISA. However, the greatest disadvantage of using culture media is the long incubation period, which can be as long as 16 weeks for a definitive diagnosis. ELISA can be performed within a few hours, although its sensitivity is estimated at only 45%, since antibodies to MAP may not be detectable in the initial stages of the infection.[6,7]

Molecular techniques to detect MAP, such as PCR, are rapid and qualitative in nature. Real-time PCR (qPCR) exhibits greater sensitivity than conventional PCR, and can determine the infective load in environmental samples, feces, milk, and cultures.[7,8] One of the target genes used to detect MAP via PCR is the IS*900* region, first described by Green et al.[9] and independently identified by Collins et al.[10] The discovery of the IS*900* region of MAP enables the diagnosis of paratuberculosis even in the initial stages of infection. The specificity and sensitivity of PCR have been enhanced up to the point of detecting 1 CFU of MAP in samples.[11]

MAP has been detected in several animal products and byproducts. A study conducted in Switzerland detected the presence of the IS*900* region in 19.7% (273/1384) milk samples collected from milk storage tanks.[12] A study in Cyprus reported 63 (28.6%) positive out of a total of 220 milk samples from tanks, using real-time PCR for IS*900* and F57.[13]

The aim of this study was to detect the IS*900* region of MAP in bovine milk samples from the State of Pernambuco (Brazil) using PCR and qPCR, and to investigate the agreement between these diagnostic tests.

Materials and methods

Sampling

In total, 121 bovine milk samples from the State of Pernambuco were collected from 6 dairy herds that already had a history of paratuberculosis. The animals were clinically healthy at the time of collection.

Sample collection and processing

Sample collection

The cow teats were cleaned with water and disinfected with 70% alcohol prior to collection of milk samples. The first 3 jets of milk were discarded. Subsequently, approximately 50 mL milk was pooled from the 4 mammary glands using sterilized and separate polypropylene tubes. The samples were stored in cool boxes containing recyclable ice and sent to the Laboratory of Bacteria in the Federal Rural University of Pernambuco for processing.

DNA extraction

DNA extraction was performed using 2 mL of each sample, which was centrifuged at 12,000 × *g* for 10 min, and the pellet was resuspended in 100 μL buffered saline solution with sterile phosphate (pH 7.2). A commercial kit[a] was used for extraction, following the manufacturer's instructions.

Polymerase chain reaction (PCR)

The extracted DNA was amplified in a final volume of 15 μL, containing the following: 5 μL genomic DNA; 0.5 μL each, of primers specific for IS*900* at 20 pM (DF: 5′-GACGACTCGACCGCTAATTG-3′ and DR-1: 5′-CCGTAACCGTCATTGTCCAG-3′); 2.75 μL ultrapure Milli-Q water and 6.25 μL PCR kit mixture,[b] as per the manufacturer instructions. The DNA was amplified in a thermocycler[c] using the following conditions: initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 3 min, with a final extension at 72 °C for 10 min.[14] The amplified product of 99 bp, corresponding to MAP, was detected by electrophoresis in agarose gel (2%), followed by staining with blue green,[d] and visualization under ultraviolet light. Images of the bands were captured.

Polymerase chain reaction in real time

The extracted DNA was amplified in a final volume of 25.0 μL containing: 5 μL genomic DNA; 1 μL each, of primers specific for IS*900* at 10 pM (DF: 5′-GACGACTCGACCGCTAATTG-3′ and the DR-1: 5′-CCGTAACCGTCATTGTCCAG-3′), 5.5 μL ultrapure Milli-Q water, and 12.5 μL real-time PCR kit mixture,[e] according to the manufacturer instructions. The DNA was amplified in a thermocycler[f] using the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s and 60 °C for 30 s. The thermocycler software was used to monitor and interpret the results.

DNA from a MAP strain provided by the National Agricultural Laboratory in Minas Gerais identified as "Nakajima 1991" was used to standardize the qPCR. The melting curve consisted of 65 °C for 90 s for preparation, with a posterior gradual increase of 1 °C every 5 s from 60 °C to 95 °C. The peak of denaturation was at 82.9 °C. The number of copies was determined as previously described by Rodrígues-Lázaro et al.[15] The copy number detected was divided by 15, which corresponds to the average number of IS*900* elements in the MAP genome.[16] The DNA of the MAP strain was 10-fold serially diluted and used to obtain the standard curve ranging from 2.27×10^7 to 22.6 MAP cells. The efficiency of the primers was 95.0% and the coefficient of linear correlation (R^2) was 0.99909, while the detection threshold (Ct) was between 9.58 and 30.08. The reactions were performed in duplicate. Finally, the number of copies in the positive field samples was quantified using the standard curve.

[a] DNA Easy Blood and Tissues Kit®, Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.
[b] MasterMix, Promega® Corp., Madison, WI.
[c] Bioer XP cycler®, Bioer Technology, Hangzhou, China.
[d] Blue Green Loading Dye (LGC Biotecnologia, São Paulo, Brazil).
[e] QuantiFast SYBR Green PCR Kit®, Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.
[f] Rotor-Gene Q thermocycler, Qiagen Biotechnology Brazil Ltda., São Paulo, Brazil.

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