Genetic analysis of MAP in bovine milk samples in Brazil

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ABSTRACT
Paratuberculosis studies has increased considerably in Brazil, however the economic impact of the disease in the country has not yet been measured. Data on the prevalence of the disease do not exist in the state of Minas Gerais, although some cases have been reported. The aim of this study was to confirm the genetic identity of MAP (Mycobacterium avium subsp. paratuberculosis) detected in raw milk samples in the region of Viçosa, MG, Brazil. A total of 220 individual bovine milk samples were analyzed by PCR using the primers BN1/BN2 derived from the insertion sequence IS900. Eight samples (3.6%) amplified fragments of the expected size, 626bp. In order to confirm the genetic identity, the eight samples were cloned, sequenced and compared with the insertion sequence IS900 deposited in GenBank (X16293.1). Out of the cloned samples, three (37.5%) and the positive control were successfully sequenced, but it was not possible to sequence the others. The genetic analysis showed 99% of identity between the sequences of this study and the sequence X16293.1, 90% with M. avium (AF527957.1), 91-92% with Mycobacterium sp. (AF455252), not-yet-identified, and 74% with Streptomyces turgidiscabies (AY707080.1) Among all the compared sequences, 11 were randomly selected to carry out a genetic sequence alignment among the 626 nucleotides, together with the four sequences of this study. From this alignment, a gene cluster map among the 15 gene sequences was built with high levels of similarity, since sequences were pooled together within the same tree branch. Only one sequence showed lower similarity and is located in another branch, probably for being a not-yet-identified Mycobacterium species. The sequencing proved that the fragments amplified in PCR reactions were MAP fragments. The results of this study allow us to affirm that MAP is present in bovine milk samples in Brazil, and it is reasonable to consider them as a first survey on the disease in the Minas Gerais State, Brazil

INTRODUCTION
Mycobacterium avium subspecies paratuberculosis (MAP) is a Gram-positive, acid-fast and facultative anaerobic, intracellular bacterium. It is a fastidious microorganism that requires the growth factor mycobactin J for in vitro growth. MAP belongs to Mycobacteriacea Family and under microscopy, usually forms small clusters (Collins, 2003). Some researchers indicate a possible role of MAP in Crohn's disease (Hermon-Taylor, 2002; Feller, et al., 2007), while others express doubt on this association (Abubakar et al., 2007; Waddell et al., 2008). Studies show that the etiology of Crohn's disease can involve a variety of viral and bacterial agents, including MAP, or an immunological origin. Evidence points to an interaction between a persistent environmental stimulus, such as a microbial antigen, and genetic factors that regulate an immunological response or a mucosal intestinal function (Shanahan and O'Mahony, 2005). The vehicle for transmission of MAP from animals to humans would be milk, but the causal association between MAP and Crohn's disease remains unclear. In the near future, the demand for milk MAP free will be a reality.

OBJECTIVES
Considering the disease socio-economical and public health significance, the significance of milk quality and presence of MAP in Brazilian milk (Carvalho et al., 2009), the aim of this study was to confirm the genetic identity of MAP detected in raw milk samples in the region of Viçosa, MG, Brazil.
MATERIALS AND METHODS

A total of 206 quarter milk samples were aseptically collected, and bulk tank milk samples from each of the 16 dairy herds in the region of Viçosa, MG, totaling 16 bulk tank milk samples, were collected. All the samples were processed and PCR was performed according to Sivakumar et al. (2005). A wild certified MAP strain was used as positive control; milli-Q water was used as negative control and φX174/HaeIII as molecular marker. In order to confirm the identity of the amplified fragments (626bp), they were cloned in the pGEM vector using the pGem T-Easy™ Vector System. Plasmid DNA was purified with Wizard® Plus SV Miniprep DNA Purification System. DNA sequencing, adapted by Sanger et al. (1977), was performed to confirm the sequences, by using the M13 forward and reverse primers. The obtained sequences were edited by the DNA MAN software and compared to IS900 (Green et al., 1989) deposited in Genbank under the accession number X16293.1, using the Basic Local Alignment Search Tool (BLAST) software, available at National Center for Biotechnology Information – NCBI website (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Eight quarter milk samples (3.6%) and none of the bulk tank milk samples amplified fragments of the expected size, 626bp (Figure 1). Out of the cloned samples, three (37.5%) and the positive control were successfully sequenced, but it was not possible to sequence the others.

The genetic analysis showed 99% of identity between the sequences of this study and the sequence X16293.1; 90% with M. avium (AF527957.1); 91-92% with Mycobacterium sp. (AF455252) not-yet-identified; and 74% with Streptomyces turgidiscabies (AY707080.1). Among the compared sequences, 11 were randomly selected to carry out a genetic sequence alignment among the 626 nucleotides, together with the four sequences of this study (Figure 2). From this alignment, a gene cluster map among the 15 gene sequences was built, showing high levels of similarity, since the sequences were pooled together within the same tree branch (Figure 3). Only one sequence showed lower similarity and is located in another branch, probably for being a not-yet-identified Mycobacterium species.

Figure 1: PCR products visualized in 1% agarose gel electrophoresis, using primer pairs BN1/BN2. 1, 2, 3, 4, 5, 6, 7 and 8) amplified samples; 9) positive control; 10) negative control: milli-Q water; M) molecular marker: φX174/HaeIII.

Figure 2: Part of the genetic alignment between DNA fragments sequenced and sequences existing in GenBank.
Figure 3: Gene cluster map between genetic sequences of MAP described in this study (MAP-UFRGS; MAP-UFV/07; MAP-UFV/09 and MAP-UFV/J) and sequences available on GenBank (X16293, AF416985, AJ250015, AJ250022, AJ250023; AJ250020, AY660658, AJ251436, AF305073, AY660657 and AF455252). Method: UPGMA; Statistical Support: 1000 replications of bootstrap.

CONCLUSION
Results confirmed presence of MAP in the analyzed samples that showed identity with the insertion sequence IS900. This study confirmed MAP in the tested milk samples, providing key information about presence of paratuberculosis in dairy herds in the region of Viçosa, Minas Gerais State, Brazil. Since there is a lack of official paratuberculosis surveys and control programs in Brazil, further studies will be needed to support the adoption of national paratuberculosis control measures. The dilution that occurs in bulk tank samples makes them non-ideal for PCR analysis, despite being the type of sample chosen to perform a herd screening. This is the first report of MAP detected in raw milk samples from dairy cattle in Brazil and it is reasonable to consider its as a first survey on the disease in the state of Minas Gerais, Brazil.

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REFERENCES


