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Occurrence of Mycobacterium avium subsp. paratuberculosis in coalho cheese in the State of Pernambuco, Brazil

[*Ocorrência de Mycobaterium avium subsp. paratuberculosis em queijo coalho do Estado de Pernambuco, Brasil*]

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ABSTRACT

Paratuberculosis is a chronic and incurable disease that affects ruminants and other domestic animals. It is caused by Mycobacterium avium subsp. paratuberculosis (MAP) that may also be involved in some human diseases such as Crohn's disease, type 1 diabetes, sarcoidosis, multiple sclerosis, and Hashimoto's thyroiditis. The objective of this study was to investigate the occurrence of MAP DNA in samples of artisanal coalho cheese purchased in the State of Pernambuco. Forty samples of coalho cheese submitted to the Real Time Polymerase Chain Reaction (qPCR) technique were analyzed for the detection of the MAP region IS900. 11 (27.5%) were positive with a mean of 195.9 MAP colony forming unit (CFU) per gram of each sample, with a minimum of 30.3 CFU/g and a maximum of 324.2 CFU/g. Thus, this type of cheese that is one of the most consumed in this region of Brazil constitutes a source of human exposure to MAP. Further research in this area should be performed to evaluate the viability of the bacteria in this cheese type.

Keywords**:** bovine, IS900, qPCR, paratuberculosis

RESUMO

Paratuberculose é uma enfermidade crônica e incurável que acomete ruminantes e outras espécies de animais domésticos. É causada pelo Mycobacterium avium subsp. paratuberculosis (MAP) e ainda há a suspeita do seu envolvimento em enfermidades nos humanos como a doença de Crohn, diabetes tipo 1, sarcoidose, esclerose múltipla e tireoidite de Hashimoto. Objetivou-se com esta pesquisa investigar a ocorrência do DNA de MAP em amostras de queijo coalho artesanal adquiridas em estabelecimentos comerciais do Estado de Pernambuco. 40 amostras de queijo coalho artesanal foram submetidas a técnica de Reação em Cadeia da Polimerase em Tempo Real (qPCR) para detecção da região IS900 do MAP. 11 (27,5%) foram positivas com uma média de 195,9 unidades formadoras de colônia (UFC) de MAP por grama de queijo, com detecção mínima de 30,3UFC/g e máxima de 324,2UFC/g. Sendo assim, esse tipo de queijo que é um dos mais consumidos nesta região do Brasil constitui uma fonte de exposição humana ao MAP. Mais pesquisas nessa área devem ser realizadas para avaliar a viabilidade dessa bactéria no queijo coalho.

Palavras-chave: bovino, IS900, qPCR, paratuberculose

INTRODUCTION

Paratuberculosis is an insidious disease that mainly affects domestic and wild ruminants and is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Kennedy and Benedictus, 2001). It is suspected that in humans, MAP may be associated with the development of some diseases, since human exposure to MAP has been identified as a potential risk factor for individuals genetically susceptible to Crohn's disease (Sechi and Dow, 2015). It has also been suspected that MAP may be involved in other

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important human diseases such as sarcoidosis (Brownell *et al*., 2011), Blau's syndrome (Dow and Ellingson, 2010), Hashimoto's thyroiditis (Sisto *et al*., 2010), multiple sclerosis (Cossu *et al*., 2011) and type 1 diabetes (Rani *et al*., 2010).

This bacterium has been reported in various types of food, such as meat, pasteurized milk, infant formula and cheese (Waddell *et al*., 2016). Due to the suspicion of MAP involvement in Crohn's disease, milk and its by-products received greater attention as a source of MAP infection for humans because they are considered to be possible carriers of this bacterium from the animal to the human (Corti and Stephan, 2002). The microorganism can be shed in the milk of infected animals, or even because raw milk can also be contaminated with fecal material containing MAP (Stabel *et al*., 2002; Donaghy *et al*., 2011).

In the northeastern region of Brazil, artisanal coalho cheese is a very popular product characterized by being a white pastry cheese with a rectangular shape and slightly acid and salty taste. Coalho cheese can be produced in considerable quantity from raw milk, which, if it is not properly hygienic, may not present microbiological safety or standardization (Almeida *et al*., 2013; Feitosa *et al*., 2003). In the State of Pernambuco, paratuberculosis has been reported in some studies involving cattle (Mota *et al*., 2007; Sá *et al*., 2013) and buffaloes (Mota *et al*., 2010) in dairy farms. The presence of DNA has also been detected in milk samples of cows from several properties in the same State (Albuquerque *et al*., 2017). Considering the risk of consumption of milk derivatives as a risk for human infection by MAP, the objective of this study was to investigate the occurrence of MAP DNA in coalho cheese samples from Pernambuco, Brazil.

METHODOLOGY

Forty samples of artisanal coalho cheese purchased in commercial establishments such as bakeries, supermarkets and public markets from Garanhuns microregion, Pernambuco, Brazil, were analyzed. The samples were sent to the laboratory in a Styrofoam box containing recyclable ice in the day of collection in their original packaging. To perform DNA extraction, 10g of cheese were weighed and macerated in 20ml of sterile 0.9% saline solution in a sterile bag. DNA extractions were performed with 20mg of the macerated material using the commercial Wizard SV Genomic DNA Purification System kit (Promega) following the manufacturer's instructions.

After DNA extractions, the Real-Time Polymerase Chain Reaction (qPCR) was performed as described by Albuquerque *et al*. (2017). It consisted of a reaction final volume of 25.0µL containing: 5µL of genomic DNA; 1µL of the specific primers for IS900 at 10µM (DF: 5'-GACGACTCGACCGCTAATTG-3 'and DR-1: 5'-CCGTAACCGTCATTGTCCAG-3') (Taddei *et al*., 2008); 5.5µL of ultra-pure mili-Q Water and 12.5µL of QuantiFast SYBR Green PCR Kit (QIAGEN® PCR mix) according to manufacturer instructions. The thermal profile of the reaction stages was performed in a Rotor-Gene Q Thermal Cycler (QIAGEN) with initial denaturation at 95°C for 5 minutes, followed by 45 cycles at 95°C for 20 seconds and 60°C for 30 seconds. The software "Rotor-Gene Q Software v1.7" was used to monitor and interpret the qPCR results.

The DNA of a MAP strain provided by the National Agricultural Laboratory of Minas Gerais identified as "Nakajima1991" was used to standardize the reaction. The melting curve consisted of 65°C for 90 seconds for the preparation, with posterior gradual increase of 0.1°C every 2 seconds from 75° to 90°C. The number of copies of the fragment was determined using methodology described by Rodríguez-Lázaro *et al*. (2005). The number of copies detected was divided by 15, which corresponds to the average copies of the IS900 region found in the MAP genome (Kralik *et al*., 2011). The MAP DNA was 10-fold serially diluted and used to obtain the standard curve, in which it was possible to detect from 21 up to 2.12 x 107 MAP cells. The efficiency of the primers was 100% and the coefficient of linear correlation (R^2) was 0.999850. The threshold value was between 9.46 and 26.43 with the denaturation peak at 83.4°C. Reactions were made in duplicates. Finally, the copy numbers from the positive samples were quantified using the standard curve obtained as reference.

To verify the presence of inhibitors in the samples, some cheese samples of 120ng/µL were

randomly selected and diluted to 100, 80, 40 and 20ng/µL of DNA. Then, 20ng of positive control were added to the diluted samples and qPCR was performed. All dilutions had the same Cq in the qPCR; therefore, there were no inhibitors in the samples. To validate the amplifications, four of the positive samples in qPCR for the IS900 region of the MAP were sent for sequencing. Samples were bidirectionally sequenced using standard protocol using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an automated ABI-PRISM 3130 sequencer (Applied Biosystems). Sequences were analyzed using the Basic Local Align Sequence Tool (BLAST; www.blast.ncbi.nlm.nih.gov/Blast.cgi) platform. This study was approved by the Ethics Committee for the Use of Animals of the Federal Rural University of Pernambuco under license no. 123/2015. The authors declare no potential conflicts of interest with respect to this study or the authorship and/or publication of this article.

RESULTS

Of the 40 cheese samples analyzed, 11 (27.5%) were positive for the IS900 region of MAP in the qPCR. In the quantification of these samples it was possible to determine an average of 195.9 MAP colony forming unit (CFU) per gram of each sample, with a minimum of 30.3 CFU/g and a maximum of 324.2 CFU/g (Figure 1). The sequencing result of the four samples exhibited a similarity ranging from 96% to 98% with the MAP sequence deposited in BLAST (MAP4 Complete Genome).

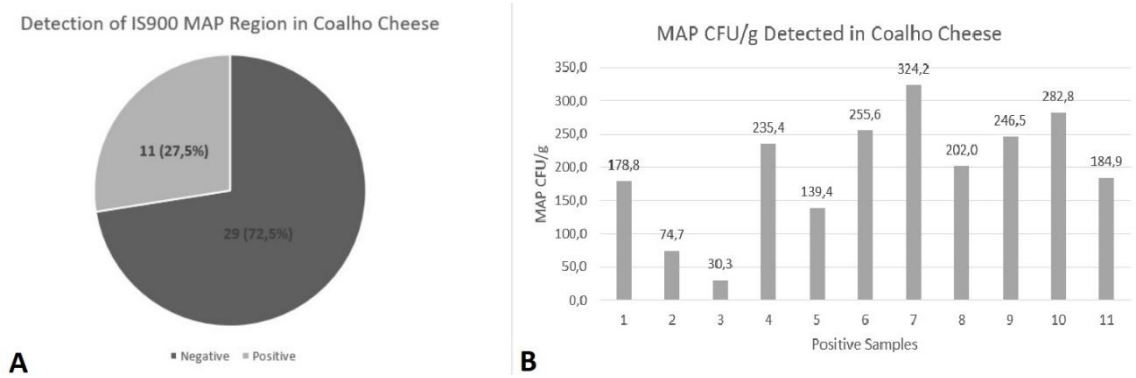

Figure 1. A - Positive samples for the IS900 MAP DNA region; B - Quantification of MAP UFC in each positive cheese sample.

DISCUSSION

The coalho cheese is a very consumed product and is part of the culture of the Northeast Region. Its manufacturing process is governed by state law, and raw fresh whole milk can be used in the artisanal confectionery of this cheese (Pernambuco, 2018). However, the use of milk without heat treatment may endanger the health of the consumer (Dias *et al*., 2015). The presence of MAP in cheeses has been previously reported in some studies worldwide (Botsaris *et al*., 2010; Williams and Withers, 2010). In Brazil, bacterial DNA was also detected in samples of coalho cheese in the State of Piauí by the PCR technique to detect the MAP region IS900, resulting in 3 (10%) positive samples (Faria *et al*., 2014).

In our study, a higher occurrence of MAP DNA was observed in coalho type cheese samples, perhaps due to the influence of the area where these samples were collected. In the state of Pernambuco, paratuberculosis has been reported with a certain frequency in cattle (Mota *et al*., 2007; Sá *et al*., 2013), and its DNA was detected in milk samples in the Garanhuns microregion (Albuquerque *et al*., 2017). It is also important to highlight that the qPCR technique used in our study has a greater sensitivity and specificity in the detection of this microorganism when compared to conventional PCR, and also has the capacity to quantify the bacteria (Mackay, 2004).

An earlier study by Albuquerque *et al*. (2017) in dairy herds from the Garanhuns microregion where some outbreaks of paratuberculosis were detected, an average of 160CFU/ml MAP was quantified in milk samples from positive cows. In this study, the number of CFU/g was higher (mean of 195.9 CFU/g), and may be justified by

the fact that the transformation of milk in to cheese basically consists of a process that concentrates a great amount of milk, in which part of the solid components, mainly protein and fat are concentrated in the curd, while the whey proteins, lactose and soluble solids are removed in the whey (Paula *et al*., 2009). And also, a study that developed an efficient procedure for isolation of MAP from raw and heat treated milk detected higher amount of CFU in the cream milk portion (Gao *et al*., 2005).

Experimentally, it has been verified that this mycobacterium can survive thermal processes that simulate pasteurization (Sung and Collins, 1998). And, an experimental study has reported that MAP microorganisms isolated from patients with Crohn's disease presented greater thermal resistance when compared to animal isolates (Chiodini and Hermon-Taylor, 1993). The coalho cheese has a pH around 5.35 and a salt concentration ranging from 0.8% to 1% (Sena *et al*., 2000) and a problem in the manufacture of this artisanal cheese is that the milk used does not need to undergo a heat treatment (Almeida *et al*., 2013). These intrinsic characteristics favor MAP survival time in cheese (Sung and Collins, 2000). In addition, the survival of this microorganism has already been reported in an isolate made from coalho cheese (Faria *et al*., 2014).

Studies involving risk factors for the development of Crohn's disease have discordant results, since some did not find an association between the microorganism and the disease in humans and others found an association between the disease and the consumption of meat and processed cheeses (Waddell *et al*., 2016). However, as animals are the main source of infection, transmission of MAP from infected animals to humans would be the most likely route (Collins, 1997).

While there is no definitive answer regarding the involvement of MAP in diseases in humans, it is important to conduct research to detect this microorganism in food, because if this relationship is confirmed, there will already be epidemiological data that may help in the control, besides, these results can still serve as indicators for possible infected herds in the region from which the samples were collected.

CONCLUSION

It is concluded that coalho cheese, which is one of the most consumed in this region of Brazil constitutes a source of human exposure to MAP. Further research in this area should be performed to evaluate the viability of the bacteria in this cheese type.

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