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First molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* identified in animal and human drinking water from dairy goat farms in Brazil

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis*, the etiologic agent of Johne's disease or paratuberculosis, was identified by culture and/or polymerase chain reaction (PCR) in 50% and 30% of water samples for animal and human consumption, respectively, from ten dairy goat farms in Brazil. IS*1311* restriction fragment length polymorphism analysis identified the isolates as cattle type C.

Paratuberculosis or Johne's disease is a chronic infectious disease, which affects mainly ruminants.[1,2] In sheep and goats generally, clinical signs appear when the animal is about one year old and include progressive weight loss, and diarrhea, that may or may not be present.[3] The etiological agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and the main elimination pathways of MAP are via feces and milk,[1,2] which contaminate pastures, milking utensils, and directly or indirectly contaminate water courses, thus infecting humans and animals. MAP is also associated frequently with Crohn's disease, given the similarities between the diseases and the frequent isolation of the bacteria in intestinal biopsies from these patients; however, the role of MAP in the pathogenesis is unclear.[4,5]

Davis et al. describes in detail the history of Crohn's disease and reports that Dalziel was the first clinician to recognize the similarity between paratuberculosis with chronic enteritis observed in humans, leading to believe that MAP was involved in the causation of the disease. But only years later, by B. B. Crohn, the disease was described during the 1930s, Although Crohn was conscious of Dalziel's notes, he did not had any success in isolating MAP from the patients, raising the controversy about the involvement of the bacteria in the pathogenesis of CD. The author even analysis that it is possibly a inaccuracy to state that the patients infected with MAP as have CD, since this is a multifactorial disease with so many unclear features, and the rejection of the medical community understand MAP as human pathogen, has had unhappy

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costs for patients infected with MAP and exposed to improper treatment.[6]

In Brazil, there a growing interest in and increased production of goat's milk, meaning that it is becoming an important alternative livestock.[6] In the Southeast region of Brazil, Minas Gerais state has the largest population of dairy goat, which is concentrated in the Zona da Mata region.[7] According to Dubeuf and Le Jaouen,[8] goat milk products have become more available to consumers since the early 2000s. Goat milk has hypoallergenic and therapeutic properties compared to cow milk[9] which has led to the increasing demand for this product and its derivatives.

Based on the comparison of the whole MAP genome, a biphasic evolution scheme has been proposed, distinguishing two main strain types: bovine (C – cattle) and sheep (S – sheep), which are genotypically and phenotypically different from each other.[10] The genetic variability of MAP has important implications for the diagnosis and control of paratuberculosis due to differences in growth rate, virulence and epidemiological characteristics.

Water is one of the most important nutrients for animals and humans, yet its importance and quality is often neglected. Water is also an important vehicle in the spread of zoonotic bacteria, and one of the major routes of contamination to animals and humans.[5,11–14] The occurrence of MAP in drinking water and even in raw water is not clear, and its presence in water samples and its role in the infection cycle remains unclear, but some studies are available, with information in other countries. Whan et al. reported the occurrence of MAP in untreated water in Northern Ireland in a viable form, and Pickup et al. identified MAP in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works in the United Kingdom, by PCR.[15–18] The subject was already researched by King et al., Chern et al., Pickup, and Beumer, but unfortunately, there were no studies available referring the occurrence of MAP in water in Brazil or any other Latin American country.[5,14,23]

Taking into consideration that water samples are non-invasive, easy to obtain and can provide the sanitary status of the herd, and also inform the health condition of the owners, the aim of this study was to verify the presence of MAP in water for human and animal consumption and determine the type of MAP strains present in the water samples from dairy goat farms in the Zona da Mata of Minas Gerais state, Brazil, one of the most important goat milk producing regions.

The study included 10 farms with a minimum of 50 animals each in order to include only establishments for commercial purposes. The farms were distributed in the seven micro-regions that comprise the Zona da Mata of the Minas Gerais meso region.

Twenty samples of 20 L of water were collected; a sample of water for animal consumption and a sample of water for human consumption from each of the ten farms. The animal samples were derived directly from water for animal consumption, collected directly from the place where the animals drank the water, while human consumption water came from the farm tap and the untreated water public system supply, or from an alternative source (well shallow artesian well/semi-artesian spring). The water for animal and human consumption was from the same source of supply in each location.

The collection vials were previously disinfected with 2.5% sodium hypochlorite, left in contact for a minimum period of 24 h, followed by washing with autoclaved distilled water.

Processing and the microbiologic culture of the samples were performed according to Pickup et al.[5] with modifications. The water sample was filtered using membrane filters of sterile cellulose (Merck Millipore, Sao Paulo, Brazil), with a 0.22 μm pore size, as many as necessary for filtration of 20 L, depending on the characteristics of the water. After filtration, the membranes were placed in 50 mL tubes, and then 30 mL 0.5× PBS, pH 6.9, was added; this was vortexed and scraped with sterile plastic loops for detachment of the retentate until the membranes were completely clean. The membranes were discarded and the retrieved content was aliquoted in 1.5 mL microtubes to perform DNA extraction and microbiological culture.

The decontamination step comprised the addition of 15 mL of 1-hexadecylpyridinium chloride 0.75% (HPC) (Sigma–Aldrich, St. Louis, MO, USA) to 1.5 mL of the sample, making contact with the sediment overnight (12–16 h). Then, the solution was centrifuged at 3000 × *g* for 20 min. After this, 2 mL of antimicrobial solution containing nalidixic acid (50 mg/L) (Sigma–Aldrich), vancomycin (50 mg/L) (Sigma–Aldrich) and amphotericin B (150 mg/L) (Critália, Itapira, Brazil) was added to the sediment, and placed in contact with the sample for 72 h at room temperature before inoculation.

Aliquots of 200 μL of each sample were inoculated into four tubes containing HEYM media, two containing mycobactin *J* and two without. The tubes were incubated at 37 °C for a minimum of 16 weeks. The MAP colonies were submitted to Ziehl-Neelsen coloring (kit staining – Laborclin), and confirmed by IS*Mav2*- PCR and genetic sequencing. MAP K10 and ultra-pure water were used as positive and negative, controls, respectively.

For DNA extraction, Wizard® Genomic DNA Purification kit was used following the manufacturer's instructions, using an initial volume of 1 mL of the sample. To perform the PCR, Go Taq® Green Master Mix kit was used according to the manufacturer's instructions, using the primer pair IS*Mav2*/F (5′-GTGAGTTGTCCGCATCAGAT-3′) and IS*Mav2*/B2 (5′-GCATCAAAGAGCACCTCGAC-3′) which amplifies a fragment of 494 bp. Each reaction had a final volume of 25 μL, with 12.5 μL mix, 1 μL of each primer, 6.5 μL nuclease free water and 4 μL of extracted DNA.[16] The amplicons were purified and sequenced in both directions in the National Agricultural Laboratory (LANAGRO) of Minas Gerais, located in Pedro Leopoldo, Brazil. The sequences obtained were aligned, edited and compared with other sequences deposited in GenBank (BLAST).

The positive samples were submitted to PCR-IS*1311* using M56 (5′-GCGTGAGGCTCTGTGGTGAA-3′) and M119 (5′-ATGACGACCGCTTGGGAGAC-3′) primers and to subsequent REA according to a previous study.[19] Enzymes *Hinf*I (Promega) and *Mse*I (NEB) were used to differentiate MAP from *M. avium* subsp. *avium*. The restriction pattern generated by *Hinf*I-digestion differentiates type S (sheep) from type C (cattle) strains, while the restriction pattern generated by *Mse*I differentiates MAP strains from *M. avium*. As controls, the K10

strain of MAP type C from cattle and VICAP711 strain of type S of sheep were used as positive controls, while nuclease free water was used as a negative control.

A sampling of farms was performed using the OpenEpi® software (available in http://www.openepi.com), considering an estimated prevalence of 5%, precision of 4% and 95% confidence interval.

MAP was identified in five farms. Four animal consumption water samples were positive by culture and PCR and a fifth sample by PCR only. In addition, three samples of water for human consumption were found to be positive to MAP by PCR in the same farms, but none were positive by microbiological culture.

Acid-Fast Bacilli were observed in slides prepared from the colonies and in the sequencing was confirmed to be MAP. The degree of identity between the DNA samples (water samples and colonies) and MAP K10 ranged from 92 to 99%.

IS*Mav*2 has low copy numbers in MAP DNA, about three to five, providing more specific results.[19–22] The MAP detection by PCR and non-observation of colonies in the same sample may be due to the type of processing, and also to the small number of bacteria present in the samples. Furthermore, MAP can form "spore-like" structures, which would hinder the growth of MAP in culture.[23,24] Nevertheless, there is the possibility that the bacteria were not viable.

It was found that all MAP strains identified in this study were type C (cattle). Research into the same properties with goat milk and fecal samples also found type C MAP strains.[22,25] In five of the visited farms, the consortium of cattle and goats were assessed, which suggested that the contamination of goats occurred by the ingestion of contaminated water or food by Map eliminated from cattle. Studies on MAP typing isolated from goats in different countries found that most strains were type C.[26–30] Type C has a wide range of hosts, and is commonly isolated from domestic and wild animals, including non-ruminants[29]; this is the predominant type in cattle.[30] Also, in patients affected by Crohn's disease, MAP isolates have also been classified as type C,[31] displaying the importance of this result.

The results indicate the possible role of water in the maintenance and dissemination of MAP in the herds. The use of this type of sample appears to be an efficient diagnostic tool in predicting the infection status of the herd. Although water plays an important role in the dissemination of infectious agents, the search for pathogens in this type of sample is uncommon.

MAP type C (cattle) DNA is present in water for human and animal consumption in dairy goat farms on properties located in the Zona da Mata de Minas Gerais. MAP was found viable in water samples for animal consumption. This is the first report of isolation and typing of MAP from water of dairy goat farms.

Conflict of interests

The authors declare no potential conflict of interests.

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