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First isolation of *Mycobacterium avium* subsp. *paratuberculosis* in a dairy goat in Argentina: Pathology and molecular characterization

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ABSTRACT

No data concerning the presence of caprine paratuberculosis (PTBC) in Argentina are currently available. In this work, a Saanen dairy goat herd located in Buenos Aires province was sampled and tested for PTBC. Feces and blood samples were collected from 210 goats. Sera were analyzed by ELISA and fecal samples were pooled for culture. Necropsy was performed in two affected adult goats. Colonies were characterized and genotyped by IS*900* PCR and IS*1311* PCR-REA, respectively. Gross pathology and histopathology of necropsied goats were consistent with PTBC. Bacteriology, serology, and PCR confirmed the diagnosis. The *Mycobacterium avium* subsp. *paratuberculosis* isolates showed a restriction pattern characteristic of type C strains. This is the first report on the isolation and molecular characterization of *M. avium* subsp. *paratuberculosis* in dairy goats in Argentina.

1. Introduction

Paratuberculosis (PTBC) or Johne's disease is a chronic, contagious and emaciating gastrointestinal disease of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map). The disease is not only troublesome for the cattle and sheep industries, but also for breeders of other ruminants, such as elk, deer, and goats, as well as for managers of captive wildlife (Ayele et al., 2001). In addition, the etiologic agent has also been implicated as a possible cause of Crohn's disease in humans (Thompson, 1994). In cattle, transmission of Map has been shown to occur by multiple routes, including the fecal–oral route, intrauterine infection, and via milk or colostrum (Sweeney et al., 1992). Infected animals may excrete Map in their feces and milk and spread the infection, which can cause considerable financial losses due to premature culling and death (Ayele et al., 2001). It is assumed that this organism infects animals in the first months of life and elicits a slowly progressive inflammatory response in the gastrointestinal tract that is not clinically evident until months to years later. Clinical signs of this infection are the same as for many other diseases: chronic weight loss and unremitting diarrhea. Diarrhea is usually absent in infected sheep and infrequent in infected goats and deer (Stehman, 1996). In advanced cases, the organism can disseminate beyond the gastrointestinal tract to other organ systems (Whitlock et al., 1997). The disease in small ruminants is widespread in the world (Harris and Barletta, 2001). In Argentina, although caprine PTBC was early reported by Ubach (1941), the etiologic agent has not been isolated yet. The present work constitutes the first report of the isolation and molecular characterization of Map in dairy goats in Argentina.

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2. Materials and methods

2.1. Population under study

Goats from a Saanen dairy goat herd located in Buenos Aires province, Argentina, were sampled and tested for PTBC as part of a research project. The herd consisted of 210 adult animals, all of which were sampled for this study. Goats grazed on pasture during the day. At night, goats were closed into the shed. Goats were milked mechanically twice daily.

2.2. Samples

Feces and blood samples were simultaneously collected once from each goat. Feces were collected *per rectum*, transferred to a sterile plastic bag and kept at −20 °C until processing. Blood samples (10 mL) were obtained from the jugular vein and, after clotting and centrifugation, the serum was harvested and frozen at −20 °C until tested. Fecal samples were pooled for culture. Pools were formed from samples of five animals of the same age group. Nineteen animals with apparent symptoms of PTBC were cultured individually.

2.3. Fecal smears and culture

Fecal smears were heat-fixed and stained by the Ziehl Neelsen's (ZN) method (OIE Manual, 2011). Culture from fecal samples was performed after applying a decontamination process. Briefly, samples were homogenized and decontaminated with hexadecylpyridinium chloride (Sigma, USA) (final concentration 0.75%) and inoculated onto three slants of Herrold's egg yolk medium (pH 7.1–7.4), with and without mycobactin J (Allied Monitor Inc., MO, Fayette, USA). The cultures were incubated at 37 °C for up to 6 months and examined biweekly for bacterial growth. Typical Map colonies were primarily characterized on the basis of mycobactin J dependency, acid fastness and slow growth. Colonies were then characterized and genotyped by IS*900* PCR and IS*1311* PCR-REA (restriction endonuclease analysis), respectively (Collins et al., 1993; Marsh et al., 1999).

2.4. IS900 PCR from cultures

PCR was used for the detection of IS*900*, as described by Collins et al. (1993). A loopful of the culture was suspended in distilled water and subjected to freeze-thaw cycles to lyse bacterial cells. The supernatant was subjected to PCR and the following conditions of amplification were used, generating a 218-bp amplification product. The mixture for the amplification consisted of: 10 mM Tris–HCl buffer, pH 9.0, 50 mM KCl and 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 250 ng of each primer, 5 μL of template, and 1.25 U of Taq polymerase (Promega, Madison, WI, USA) and water to a final volume of 50 μL. PCR products were analyzed by submerged electrophoresis on a 2% agarose gel.

2.5. IS1311 PCR-REA

In order to identify the subspecies of *M. avium* isolated and strain type, PCR and PCR-REA of IS*1311* gene were performed. The protocol according to Marsh et al. (1999) was used. Briefly, the IS*1311* was amplified using the primers M56 and M119 under the following conditions: one cycle of denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 15 s and extension at 72 °C for 1 min. The 608-bp product was digested using 2 U of *Hinf* (NEB) and *Mse*I (NEB) using the buffer provided by the manufacturer and adding 1.6 μL of 100 μg/mL bovine serum albumin per reaction. The restriction products were resolved in 4% agarose gels stained with ethidium bromide. The presence of 67-bp, 218-bp, 285-bp and 323-bp products is indicative of type C strains, while the presence of restriction fragments of 285 bp and 323 bp is indicative of type S strains.

2.6. Pathological examination

After euthanasia, necropsy was performed in two affected adult goats (#1 and #2). Samples of the ileocecal valve, ileum, jejunum, mesenteric lymph node (MLN), and cecum were collected for histopathological examination and culture. Samples for histopathology were fixed in 10% buffered formalin and processed following routine procedures. Sections (2 μm in thickness) were cut from each sample and stained with hematoxylin and eosin (HE) and by the ZN method for acid-fast bacteria (AFB). Sections of the ileum and MLN were used for immunohistochemistry. The technique was performed following procedures previously described (Delgado et al., 2009).

The smears were heat-fixed and stained with the ZN method. The presence of clumps of three or more strongly AFB was recorded as positive (OIE Manual, 2011).

Tissue samples for bacteriological examination were homogenized with 5 mL sterile physiological saline and processed as described for fecal culture.

2.7. Serology

The sera of all the animals were analyzed by indirect enzyme immunosorbent assay (ELISA) using Map protoplasmic antigen (PPA-3; Allied Monitor, Fayette, MO, USA). The indirect ELISA technique used was a slight modification of that described by Verna et al. (2007). Briefly, the antigen was diluted to a concentration of 0.07 mg/mL in carbonate buffer and a volume of 100 μL per well was allowed to fix overnight at 4 °C in flat-bottomed polystyrene plates (Immulon I, Dynatech, USA). To eliminate unspecific antibodies, sera were previously absorbed with a 1:1 suspension of *Mycobacterium phlei* for 2 h at room temperature under stirring and then centrifuged. After centrifugation, the supernatant fraction of each sample was recovered and diluted 1:100 in phosphate-buffered-Tris–glycerine solution (PBS-TG) and then 100 μL was added to each well. Each sample was tested in duplicate. The plates were then incubated for 2 h at room temperature. After washing with PBS-TG, a 1:1500 dilution of a horseradish peroxidase-labeled Protein G (Bio-Rad Laboratories, Hercules, California, USA) was added to each well. After 2 h of incubation at room temperature, the plates were washed thoroughly, and 100 μL of substrate solution (0.04 M ABTS in citrate buffer (0.05 M pH 5) and 1 mM H_2O_2 100 vol) was added to each well. After 10 min of incubation at room temperature, the A450 was recorded with a microplate reader (Titertek, Multiskan, ICN, Finland). The cut-off value for a positive test was based on the absorbance values at 450 nm of 326 serum samples from goats testing negative to the Johnin test, fecal culture and from a region of Argentina where no animals with symptoms of PTBC have been observed. Samples having at least twice the mean absorbance of the negative serum were recorded as positive (Rajukumar et al., 2001). Accordingly, the optical density (OD) value of 0.375 was fixed as the cut-off value for deciding the positive sera in the ELISA. The OD results were transformed to an index value by division of the mean OD obtained with the serum samples from the animals by the mean OD of the negative controls.

3. Results and discussion

The clinical signs reported in the goats studied in this work were mainly those of a chronic wasting disease which leads to emaciation, severe weakness, prostration and death and the disease was characterized mainly by a progressive loss of weight but without diarrhea.

Culture is considered the only definitive and critical technique to diagnose Johne's disease (Whittington et al., 1999). Pooling sheep fecal samples has been reported by Australian investigators as an efficient method to detect PTBC in flocks and has been used as an excellent tool to monitor sheep flocks over time. This same approach could also be used for deer and goat flocks (Whitlock, 2005). In agreement with these findings, in our work, 15 out of 34 (44.1%) pools of fecal samples tested in the infected herd were culture-positive. The use of pooled fecal samples was a useful tool, since Map excretion was demonstrated in at least 15 out of 170 (8.8%) animals without clinical signs. The highest number of positive pools was that of the animals aged 4 years (Table 1). Nevertheless, clinical signs and fecal culture-positive samples were also observed in 1-year-old goats. Typically, sheep and goats are 2–4 years old when