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Case Report

Mycobacterium paratuberculosis sheep type strain in Uruguay: Evidence for a wider geographic distribution in South America

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Abstract

Johne's disease (JD) is an economically important disease of ruminants caused by *Mycobacterium avium paratuberculosis* (MAP), which also infects other species including humans. Two major MAP strain types are currently recognized: sheep (S) and cattle (C) types. Information on JD prevalence and MAP types infecting small ruminants in South America is limited, and all but one of the MAP types reported from this region are of the C type. This study describes clinicopathological, molecular and microbiological findings in 11 cases of JD caused by a type S MAP strain, and estimated true within-flock prevalence in a ~735-sheep operation in Uruguay. Postmortem examination and histology (hematoxylin-eosin and Ziehl-Neelsen stains) of samples from 41 selected sheep revealed lymphohistiocytic/granulomatous enteritis and mesenteric lymphadenitis in 11 animals, with moderate/severe multibacillary lesions in 6 clinical cases, and minimal/mild paucibacillary lesions in 5 sub-clinical cases. Immunohistochemistry using an antibody against *Mycobacterium bovis* that cross-reacts with MAP (2 cases), and transmission electron microscopy (1 case), revealed myriads of intrahistiocytic mycobacteria. MAP was isolated in one case and detected by PCR in 6 cases. The S type of MAP was identified using a multiplex PCR that distinguishes between S and C types, and PCR-REA. The estimated true within-flock prevalence was ≤ 2.3%. This represents the first communication on within-flock prevalence of JD associated with a type S MAP strain in South America and the second documentation of this strain in the subcontinent. Additional studies are required to better understand the molecular epidemiology of the different MAP types in the region.

Key words: enteritis; infectious disease; livestock; paratuberculosis; ruminants; South America.

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Introduction

Johne's disease (JD) is a slowly-progressing disease caused by *Mycobacterium avium paratuberculosis* (MAP), characterized by chronic diarrhea and granulomatous enteritis. JD affects domestic and wild ruminants worldwide [1-3], and is responsible for large economic losses to the livestock industry [4]. Although not typically considered zoonotic, MAP can infect humans [5] and has been associated with Crohn's disease in this species [1,6].

With the advancement of molecular techniques, various MAP strains with different potential for disease development have been identified. The 2 major strain groups currently recognized are the sheep- (S-) and cattle- (C-) types, named after the species from which they were first isolated [7,8]. Although there are remarkable genomic differences between these types [8,9], both have been shown to cause JD in sheep [10,11].

Although JD is widespread in Uruguayan dairy cattle, it had not been recognized in Uruguayan sheep, and there are no reports of the MAP strain types in any species from this country. This study aims to describe the clinicopathological, epidemiological, and microbiological findings in 11 cases of ovine JD (OJD) in a sheep operation in Uruguay, broadening the current knowledge on geographic distribution and within-flock prevalence of S-type MAP in South America (SA).

Case Report

Clinical signs and pathological findings (autopsy, histology, immunohistochemistry and electron microscopy)

The cases occurred at a 735-sheep flock in Colonia, Uruguay, managed under extensive grazing conditions. Six clinically-affected sheep (A-F) were examined between May-2015 and March-2016. Clinical signs included weight loss, emaciation, intermittent diarrhea and/or submandibular edema. Carcasses of these sheep (A-F) were subjected to full autopsies. In April-2016, 35 sheep from this flock, were sent to the slaughterhouse. The ileum and mesenteric lymph nodes (MLN) of these animals were examined grossly. Fresh MLN/ileum and feces were collected and stored. Ileum and MLN from all 41 animals were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin and Ziehl-Neelsen (ZN) stains for histologic examination. Additionally, ileum and MLN from cases A-B were processed for immunohistochemistry using a polyclonal antibody against Bacille Calmette-Guerin (BCG), an attenuated *Mycobacterium bovis* strain, that cross-reacts with other mycobacteria [12,13]. Briefly, epitope unmasking was accomplished by autoclaving the slides for 10 minutes at 121°C in citrate buffer (Biocare Medical, CB910M, Concord, CA), followed by a 10-minute blocking step using a casein buffer (Biocare Medical, BP974M, Concord, CA). A polyclonal antibody against BCG produced in rabbit (Dako B0124, Carpinteria, CA) was used as a primary antibody for 30 min at a 1:3000 dilution. An anti-rabbit horseradish peroxidase (HRP)-labelled polymer (Dako K4003, Carpinteria, CA) was used as a detecting antibody for 30 min. Antibody binding was visualized using 3-amino-9-ethylcarbazol (AEC) substrate chromogen (Dako K3464, Carpinteria, CA). Appropriate positive and negative controls were used.

Autopsy revealed mesenteric lymphangiectasia, and/or moderate diffuse thickening of the ileum, with roughened mucosal surface (Figure 1) in all cases evaluated (A-F). Of the 35 animals examined at the slaughterhouse, 3 (G-I) had slightly increased mucosal plicae in the ileum. Histologically in cases A-F, there was moderate/severe diffuse granulomatous enteritis (Figure 2), lymphangitis and mesenteric lymphadenitis. Infiltrating macrophages in the intestines and MLN contained myriads of intracytoplasmic ZN-positive bacilli (multibacillary lesions) (Figure 3) which was accompanied by strong granular immunoreactivity with anti-BCG antibody in cases A-B, the only 2 cases evaluated by this technique. In cases G-I, and in 2

Figure 1. Sheep C, autopsy findings. The mucosa of the ileum is moderately and diffusely thickened with a roughened and undulated surface, typical of granulomatous enteritis.

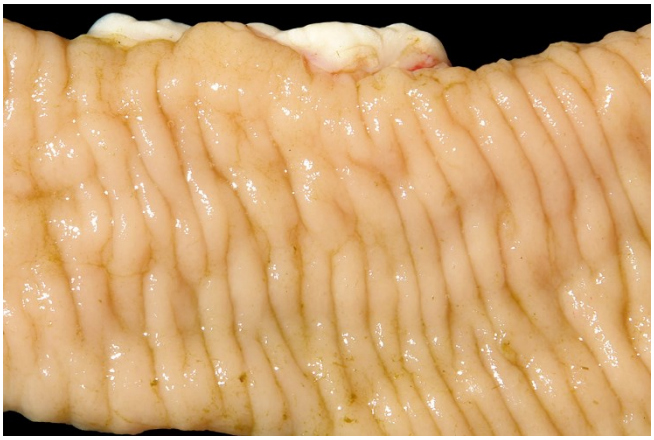


Figure 2. Sheep C, ileum, histology. Severe granulomatous enteritis characterized by numerous macrophages infiltrating the lamina propria and separating the intestinal crypts. Hematoxylin and eosin stain, bar= 50 µm.

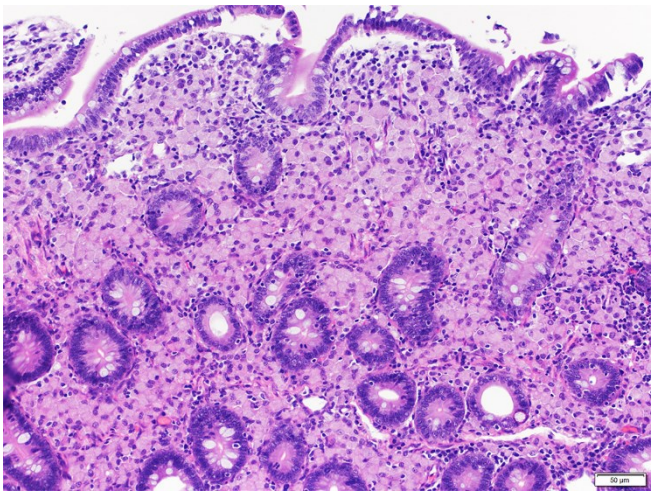
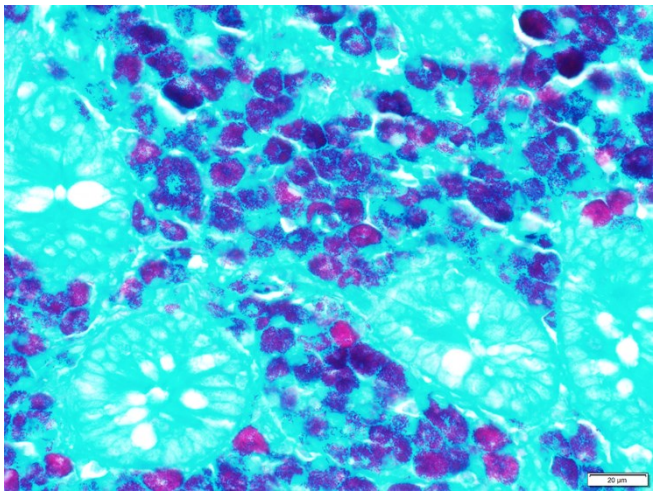


Figure 3. Sheep C, ileum, histology. Infiltrating macrophages in the lamina propria contain myriads of intracytoplasmic ZN positive bacilli (multibacillary lesion). Ziehl-Neelsen stain, bar= 20 µm.



animals that did not have gross lesions (J-K), the microscopic examination revealed, respectively, mild and minimal lymphohistiocytic ileitis and mesenteric lymphadenitis with rare intrahistiocytic ZN-positive bacilli (paucibacillary lesions).

Transmission electron microscopy was performed on formalin-fixed ileum from sheep B [14]. Macrophages infiltrating the ileum had large numbers of intracellular mycobacteria, with cross and sagittal/tangential sections of structurally-intact bacilli within phagocytic vacuoles or free in the cytosol (Figure 4).

MAP culture and molecular testing

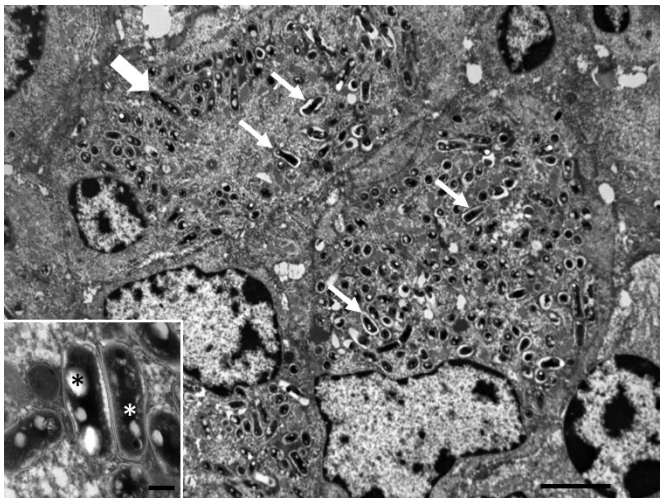
Ileum from case E was processed for MAP culture in Middlebrook-7H9 liquid medium supplemented with mycobactin-J (Allied Monitor), albumin-dextrose-catalase growth supplement (Middlebrook ADC), casitone, egg yolk and an antibiotic mixture (BACTEC-MGIT-960, Becton-Dickinson) [15,16]. MAP was isolated after 2 months of incubation.

Paraffin-embedded ileum from cases A-B were processed for MAP detection by real-time PCR using VetMAX MAP Reagents following DNA extraction with RecoverAll™ (ThermoFisher). MAP was detected in both cases (ct=24.98 and 24.25). Furthermore, DNA from frozen ileum and/or feces from cases C-F was extracted with a commercial kit (ZR-Fecal-DNA-Miniprep, ZymoResearch). MAP was detected in all 4 cases by amplifying a fragment of the insertion sequence IS*900* with primers DirC and RevC [17]. MAP subtyping was performed in cases C-D by a multiplex PCR that distinguishes S/C types, targeting the IS*900* (primers DMC529, DMC531, and DMC533) [18]. In addition, IS*1311* PCR-restriction enzyme analysis (PCR-REA) was performed on DNA extracted from ileum in case C, and the MAP strain cultured from case E [19]. Band patterns after PCR-REA were compared with control DNA from MAP C-strain K10 and MAP S-strain S397 [8]. Genetic typing in the 3 cases was consistent with S-type MAP.

Serology

Serum from 244/735 sheep in the flock, selected by systematic sampling (one every three sheep), was obtained on December-2015, and processed using an ELISA kit (Paracheck-2, Life Technologies). Anti-MAP antibodies were detected in 3/244 (1.23%) animals. A within-flock true prevalence of ≤ 2.3% was calculated based on the ELISA's sensitivity and specificity, using methods previously described [20].

Figure 4. Sheep B. Ultramicrograph of macrophages infected with MAP in the lamina propria of the ileum. A longitudinal section of a mycobacteria free in the cytosol is indicated with a thick arrow. Bacteria within phagocytic vacuoles are marked with thin arrows. Bacteria had an average diameter of approximately 0.3 µm. Bar= 2 µm, original magnification 7500×. Inset: higher detail of mycobacteria free in the cytosol, note an outer and inner membrane, nucleoid (white asterisk) and lipid droplets (black asterisk). Bar= 0.2 µm, original magnification 120000×. Transmission electron microscopy.



Discussion

The epidemiologic characteristics of the outbreak, the clinical signs and pathological findings in the affected animals strongly supported a diagnosis of JD. Enteric mycobacteriosis was confirmed by ZN stain and anti-BCG immunohistochemistry, and MAP was isolated, detected by PCR, and typed as an S type, allowing for definitive etiologic confirmation of OJD, a disease previously unreported in Uruguay.

We speculate that OJD had been present in Uruguay for years without being recognized. Unlike the clinical disease in cattle, which is characterized by profuse diarrhea, affected sheep display progressive weight loss, with soft feces, but usually not severe diarrhea, in some animals [21]. The lack of diarrhea in sheep makes clinical disease recognition more challenging, particularly in flocks in extensive production systems, which preclude routine close monitoring of body weight.

The most definitive diagnostic test for confirmation of OJD is postmortem examination of the intestines, with histologic demonstration of the lesions and MAP by specific staining [22-24]. Another approach is routine surveillance of sheep at slaughterhouses, a method used extensively to detect new cases of OJD in Australia [22]. This approach allowed us to identify additional sub-clinically infected animals, and could be implemented in Uruguay to cost-effectively monitor the prevalence of MAP in sheep populations.

Based on the presence and type of lesions and the concentration of intralesional MAP bacilli, three forms of OJD have been recognized: multibacillary disease, paucibacillary disease, and asymptomatic infection [24,25]. Multibacillary lesions are characterized by severe granulomatous enteritis, with infiltrating macrophages containing numerous intracytoplasmic MAP bacilli. Paucibacillary lesions are more lymphocytic in nature, with intralesional bacilli being far less numerous. The histologic distribution and severity of the lesions, and the large numbers of intralesional MAP observed with ZN stain and/or anti-BCG IHC in this report were consistent with the multibacillary form in 6 cases. Five cases had paucibacillary lesions. Since animals with multibacillary lesions may be high fecal shedders and eliminate on average 10^{10} MAP microorganisms per day, this higher proportion of animals with multibacillary lesions may have favored environmental contamination with MAP in the farm.

The immunohistochemistry technique using a primary antibody against MAP has been used for the identification of MAP in formalin-fixed intestine in infected cattle. With the same purpose in this work, we used a primary antibody against BCG for the detection of MAP in ovine intestines and MLN. The cross-reactivity of polyclonal antibodies against BCG has been used in previous studies for the intralesional detection of other bacteria, including non-*M. bovis* mycobacteria, by immunohistochemistry in humans and animals [12,13]. However, to the best of our knowledge, this is the first time that an anti-BCG immunohistochemistry technique was used for the observation of MAP in ovine tissues, with positive results.

Using multiplex PCR and PCR-REA, we typed the MAP infecting these animals as a type-S strain. Although this type is commonly found in sheep in Australia [21] and Europe [7], nearly all reports on molecular typing of MAP from SA sheep, cattle, goats, deer, alpacas, guanacos, hares, bovine milk, and environmental samples, including Argentina, Brazil, Chile, Colombia and Venezuela [2,3,10,26-31], and the Caribbean [32], have been of the C type. MAP S-type has only been described once in SA (Argentina) in a single case of OJD in a Milchschaf ram [11]. Although the genotyping method we used targeting IS*1311* is one of the most widely applied methodologies for MAP typing, along with mycobacterial interspersed repetitive units-variable numbers of tandem repeats (MINRU-VNTR), these methods have limitations when compared to genome-wide single nucleotide polymorphism (SNP) and phylogenetic analyses [7]. Given the importance of genetic diversity in understanding the epidemiology of MAP, whole genome sequencing of South American MAP strains should provide information to better understand transmission and pathogenic potential.

Recently published systematic reviews of JD in ruminants in Latin America and the Caribbean concluded that currently available studies are insufficient to determine the prevalence of JD in farmed ruminants in the region, and specifically identified the lack of information from Uruguay [33,34]. To assess the seroprevalence of JD in this flock, we used a commercial ELISA, and estimated a true within-flock prevalence $\leq 2.3\%$. In addition, we identified a total of 11 infected animals (1.5% of the flock) by means of pathologic examination coupled with ZN stain in 41 animals examined postmortem. This represents a situation comparable to a low to medium within-flock prevalence for OJD in Australia, where OJD has been extensively studied [35]. No studies on within-flock prevalence of OJD caused by S-type MAP are available from SA, where the only report of S type MAP described an individual case of OJD [11]. More extensive epidemiological studies are needed to investigate the prevalence and distribution of OJD in the region.

In conclusion, OJD is present in Uruguay, which may have negative economic consequences for the livestock industry. This represents the second documentation of S-type MAP in SA, and the first report on within-flock JD prevalence associated with this MAP type in the subcontinent. Additional studies are required to better understand JD geographic distribution, economic impact, and molecular epidemiology of the different MAP strains infecting animals in the region.

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