

## Case Report

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

Federico Giannitti<sup>1,2</sup>, Martín Fraga<sup>1</sup>, Rubén Darío Caffarena<sup>1</sup>, Carlos Omar Schild<sup>1</sup>, Georget Banchemo<sup>1</sup>, Aníbal Guillermo Armien<sup>2</sup>, Gabriel Travería<sup>3</sup>, Douglas Marthaler<sup>2</sup>, Scott Joseph Wells<sup>2</sup>, Franklin Riet-Correa<sup>1</sup>

<sup>1</sup> Instituto Nacional de Investigación Agropecuaria (INIA), La Estanzuela, Colonia, Uruguay

<sup>2</sup> Veterinary Population Medicine Department, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States

<sup>3</sup> Centro de Diagnóstico e Investigaciones Veterinarias (CEDIVE), Universidad Nacional de La Plata, Chascomús, Buenos Aires, Argentina

### 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  $\leq 2.3\%$ . 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.

*J Infect Dev Ctries* 2018; 12(3):190-195. doi:10.3855/jidc.9751

(Received 11 September 2017 – Accepted 15 March 2018)

Copyright © 2018 Giannitti *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

### 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)*

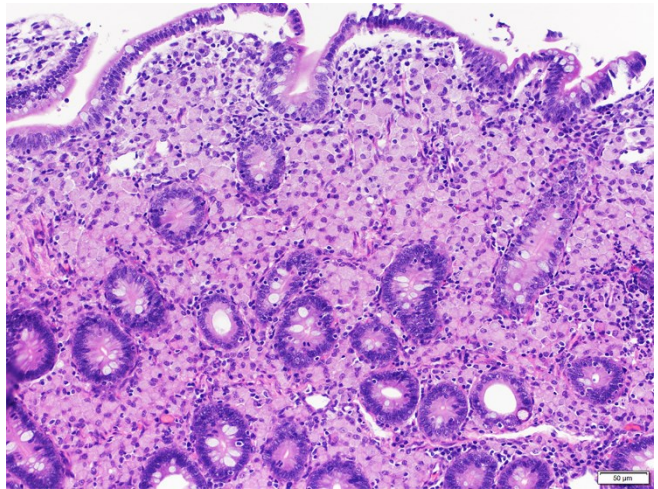
The cases occurred at a 735-sheep flock in Colonia, Uruguay, managed under extensive grazing conditions. Six clinically-affected sheep (A-F) were examined between May-2015 and March-2016. Clinical signs included weight loss, emaciation, intermittent diarrhea and/or submandibular edema. Carcasses of these 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

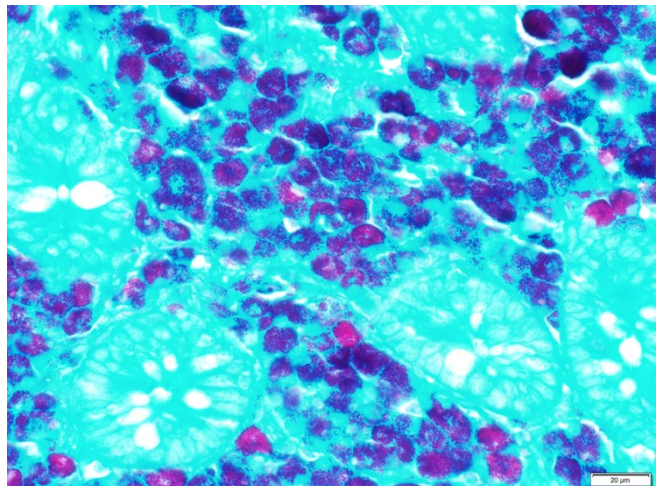
**Figure 1.** Sheep C, autopsy findings. The mucosa of the ileum is moderately and diffusely thickened with a roughened and undulated surface, typical of granulomatous enteritis.



**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

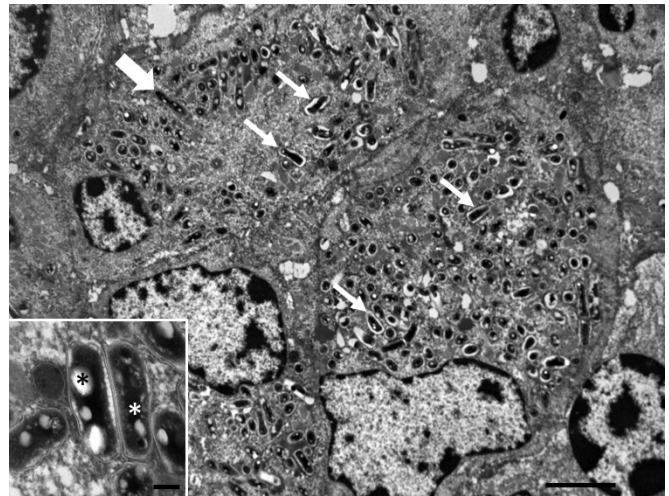
Ileum from case E was processed for MAP culture in Middlebrook-7H9 liquid medium supplemented with mycobactin-J (Allied Monitor), albumin-dextrose-catalase growth supplement (Middlebrook ADC), casitone, egg yolk and an antibiotic mixture (BACTEC-MGIT-960, Becton-Dickinson) [15,16]. MAP was isolated after 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 IS900 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 IS900 (primers DMC529, DMC531, and DMC533) [18]. In addition, IS1311 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  $\leq 2.3\%$  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  $\mu\text{m}$ . Bar= 2  $\mu\text{m}$ , original magnification 7500 $\times$ . 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  $\mu\text{m}$ , original magnification 120000 $\times$ . 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 Milchschaaf ram [11]. Although the genotyping method we used targeting IS/311 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.

### Acknowledgements

The authors thank Yisell Perdomo, Cecilia Monesiglio and Laura Casaux (Instituto Nacional de Investigación Agropecuaria, Uruguay), Karen Sverlow (University of California, Davis), and Dr. Srinand Sreevatsan (Veterinary Population Medicine Department, University of Minnesota) for technical and laboratory assistance.

### References

- 1- Romero C, Hamdi A, Valentine JF, Naser SA (2005) Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subspecies *paratuberculosis* DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm Bowel Dis* 11: 116–125.

- 2- Salgado M, Herthnech D, Bölske G, Leiva S, Kruze J (2009) First isolation of *Mycobacterium avium* subsp. *paratuberculosis* from wild guanacos (*Lama guanicoe*) on Tierra del Fuego Island. J Wildl Dis 45: 295–301.
- 3- Salgado M, Monti G, Sevilla I, Manning E (2014) Association between cattle herd *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection and infection of a hare population. Trop Anim Health Prod 46: 1313–1316.
- 4- Garcia AB, Shalloo L (2015) Invited review: The economic impact and control of paratuberculosis in cattle. J Dairy Sci 98: 5019–5039.
- 5- Richter E, Wessling J, Lügering N, Domschke W, Rüscher-Gerdes S (2002) *Mycobacterium avium* subsp. *paratuberculosis* infection in a patient with HIV, Germany. Emerg Infect Dis 8: 729–731.
- 6- Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M (2007). *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. Lancet Infect Dis 7: 607–613.
- 7- Bryant JM, Thibault VC, Smith DG, McLuckie J, Heron I, Sevilla IA, Biet F, Harris SR, Maskell DJ, Bentley SD, Parkhill J, Stevenson K (2016) Phylogenomic exploration of the relationships between strains of *Mycobacterium avium* subspecies *paratuberculosis*. BMC Genomics 17: 19.
- 8- Stevenson K (2015) Genetic diversity of *Mycobacterium avium* subspecies *paratuberculosis* and the influence of strain type on infection and pathogenesis: a review. Vet Res 46: 64.
- 9- Bannantine JP, Wu C, Hsu C, Zhou S, Schwartz DC, Bayles DO, Paustian ML, Alt DP, Sreevatsan S, Kapur V, Talaat AM (2012) Genome sequencing of ovine isolates of *Mycobacterium avium* subspecies *paratuberculosis* offers insights into host association. BMC Genomics 13: 89.
- 10- Gioffré A, Correa Muñoz M, Alvarado Pinedo MF, Vaca R, Morsella C, Fiorentino MA, Paolicchi F, Ruybal P, Zumarraga M, Traveria GE, Romano MI (2015) Molecular typing of Argentinian *Mycobacterium avium* subsp. *paratuberculosis* isolates by multiple-locus variable number-tandem repeat analysis. Braz J Microbiol 46: 557–564.
- 11- Traveria GE, Zumarraga M, Etchehoury I, Romano MI, Cataldi A, Pinedo MF, Pavlik I, Pribylova R, Romero JR (2014) First identification of *Mycobacterium avium paratuberculosis* sheep strain in Argentina. Braz J Microbiol 44: 897–899.
- 12- Bonenberger TE, Ihrke PJ, Naydan DK, Affolter VK (2001) Rapid identification of tissue micro-organisms in skin biopsy specimens from domestic animals using polyclonal BCG antibody. Vet Dermatol 12: 41–47.
- 13- Wiley EL, Mulhollan TJ, Beck B, Tyndall JA, Freeman RG (1990) Polyclonal antibodies raised against Bacillus Calmette-Guerin, *Mycobacterium duvalii*, and *Mycobacterium paratuberculosis* used to detect mycobacteria in tissue with the use of immunohistochemical techniques. Am J Clin Pathol 34: 307–312.
- 14- Phelps NBD, Mor SK, Armién AG, Pelican KM, Goyal SM (2015) Description of the microsporidian parasite, *Heterosporis sutherlandae* n. sp. infecting fish in the Great Lakes region, USA. PLoS One 10: e0132027.
- 15- Whittington RJ, Marsh I, McAllister S, Turner MJ, Marshall DJ, Fraser CA (1999) Evaluation of a modified BACTEC 12B radiometric medium and solid media for culture of *Mycobacterium avium* subsp. *paratuberculosis* from sheep. J Clin Microbiol 37: 1077–1083.
- 16- Whittington RJ, Whittington AM, Waldron A, Begg DJ, de Silva K, Purdie AC, Plain KM (2013) Development and validation of a liquid medium (M7H9C) for routine culture of *Mycobacterium avium* subsp. *paratuberculosis* to replace modified Bactec 12B medium. J Clin Microbiol 51: 3993–4000.
- 17- Collins DM, Stephens DM, De Lisle GW (1993) Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. Vet Microbiol 36: 289–299.
- 18- Collins DM, De Zoete M, Cavaignac SM (2002) *Mycobacterium avium* subsp. *paratuberculosis* strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference. J Clin Microbiol 40: 4760–4762.
- 19- Motiwala AS, Strother M, Amonsin A, Byrum B, Naser SA, Stabel JR, Shulaw JR, Bannantine JP, Kapur V, Sreevatsan S (2003) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis*: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. J Clin Microbiol 41: 2015–2026.
- 20- Rogan WJ, Gladen B (1978) Estimating prevalence from the results of a screening test. Am J Epidemiol 107: 71–76.
- 21- Windsor PA (2015) Paratuberculosis in sheep and goats. Vet Microbiol 181: 161–169.
- 22- Windsor PA (2014) Managing control programs for ovine caseous lymphadenitis and paratuberculosis in Australia and the need for persistent vaccination. Vet Med Res Reports 5: 11–21.
- 23- Pérez V, García Marín JF, Badiola JJ (1996) Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. J Comp Pathol 114: 107–122.
- 24- Sonawane GG, Tripathi BN (2016) Comparative evaluation of diagnostic tests for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in the tissues of sheep affected with distinct pathology of paratuberculosis. Int J Mycobacteriol 5 Suppl 1: 88–89.
- 25- Dennis MM, Reddacliff LA, Whittington RJ (2011) Longitudinal study of clinicopathological features of Johne's Disease in sheep naturally exposed to *Mycobacterium avium* subspecies *paratuberculosis*. Vet Pathol 48: 565–575.
- 26- Carvalho IA, Pietralonga PA, Schwarz DG, Faria AC, Moreira MA (2012) Short communication: Recovery of viable *Mycobacterium avium* subspecies *paratuberculosis* from retail pasteurized whole milk in Brazil. J Dairy Sci 95: 6946–6948.
- 27- Fernández-Silva JA, Abdulmawjood A, Akineden Ö, Bülte M (2012) Genotypes of *Mycobacterium avium* subsp. *paratuberculosis* from South American countries determined by two methods based on genomic repetitive sequences. Trop Anim Health Prod 44: 1123–1126.
- 28- Fiorentino MA, Gioffré A, Cirone K, Morsella C, Alonso B, Delgado F, Paolicchi F (2012) First isolation of *Mycobacterium avium* subsp. *paratuberculosis* in a dairy goat in Argentina: Pathology and molecular characterization. Small Ruminant Res 108: 133–136.
- 29- Moreira AR, Paolicchi F, Morsella C, Zumarraga M, Cataldi A, Fabiana B, Alicia A, Piet O, van Soelingen D, Isabel RM (1999) Distribution of IS900 restriction fragment length polymorphism types among animal *Mycobacterium avium* subsp. *paratuberculosis* isolates from Argentina and Europe. Vet Microbiol 70: 251–259.

- 30- Salgado M, Sevilla I, Rios C, Crossley J, Tejada C, Manning E (2016) Presence of *Mycobacterium avium* subsp. *paratuberculosis* in alpacas (*Lama pacos*) inhabiting the Chilean Altiplano. *J Zoo Wildl Med* 47: 12–16.
- 31- Souza MCC, Lima MC, Braga IFE, Schwarz DGC, Rodrigues APS, Sales EB (2016) Molecular typing of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolated from dairy goats in Brazil. *Small Ruminant Res* 140: 18–21.
- 32- Kumthekar S, Manning EJ, Gosh P, Tiwari K, Sharma RN, Hariharan H (2013) *Mycobacterium avium* subspecies *paratuberculosis* confirmed following serological surveillance of small ruminants in Grenada, West Indies. *J Vet Diagn Invest* 25: 527–530.
- 33- Fernández-Silva JA, Correa-Valencia NM, Ramírez NF (2014) Systematic review of the prevalence of paratuberculosis in cattle, sheep, and goats in Latin America and the Caribbean. *Trop Anim Health Prod* 46: 1321–1340.
- 34- Espeschit IF, Schwarz DGG, Faria ACS, Souza MCC, Paolicchi FA, Juste RA, Carvalho IA, Moreira MAS (2017) Paratuberculosis in Latin America: a systematic review. *Trop Anim Health Prod* 48: 1557–1576.
- 35- Dhand NK, Eppleston J, Whittington RJ, Toribio JA (2007) Risk factors for ovine Johne's disease in infected sheep flocks in Australia. *Prev Vet Med* 82: 51–71.

**Corresponding author**

Federico Giannitti  
Ruta 50 km 11, La Estanzuela  
(70006) Colonia, Uruguay  
Phone: +59845748000,  
Fax: +59845748012.  
E-mail: fgiannitti@inia.org.uy , fgiannit@umn.edu

**Conflict of interests:** No conflict of interests is declared.