Brazilian Journal of Microbiology 44, 3, 897-899 (2013)
ISSN 1678-4405

Short Communication

First identification of *Mycobacterium avium paratuberculosis* sheep strain in Argentina

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Submitted: May 15, 2011; Approved: September 10, 2012.

Abstract

We here identified for the first time the presence of *Mycobacterium avium paratuberculosis* (MAP) sheep (S) strain in Argentina. IS*900* polymerase chain reaction (PCR) was positive. The S strain was compared with MAP cattle (C) strains by using IS1311 PCR-restriction endonuclease analysis (PCR-REA), multiplex PCR and restriction fragment length polymorphism (RFLP) analysis.

Key words: paratuberculosis, sheep, typing, PCR, PCR-REA.

Paratuberculosis is a chronic proliferative enteritis of domestic and wild ruminants caused by *Mycobacterium avium paratuberculosis* (MAP). In small ruminants, progressive loss of weight is the most common clinical sign of the disease, but unlike cattle, clinically affected sheep not always present diarrhoea. Animals are usually infected through the faecal-oral route and it can take years before the onset of the clinical disease occurs (Sweeney *et al.*, 1996). Based on restriction fragment length polymorphisms (RFLP) analysis, MAP strains have been classified into cattle (C), sheep (S) and intermediate (I) types (Collins *et al.*, 1990; De Lisle *et al.*, 1992; Pavlik *et al.*, 1995, 1999). Sheep tend to be infected by the S strains (Whittington *et al.*, 2000), which are characterised by culture requirements different from those of the C strains. By pulsed field gel electrophoresis (PFGE), MAP strains can also be divided into type I (sheep strain), type II (cattle strain) and type III (a sub-type of type I also known as intermediate strain) (De Juan *et al.*, 2005; Stevenson *et al.*, 2002). PCR-based methods, such as IS*1311*-PCR/REA targeting IS*1311* after digestion using *Hinf*I restriction nuclease (Marsh *et al.*, 1999) or multiplex DMC-PCR assay, can also distinguish between C (type II) and S (type I/III) isolates (Collins *et al.*, 2002). Since the first description of a sheep with paratuberculosis in Argentina (Ault, 1942), there have been no mentions on differences between isolates of MAP from sheep and cattle (Jorge *et al.*, 2000). In the present work, blood was collected by jugular venipuncture from one sheep prior to euthanasia by intravenous injection of a sodium pentobarbital overdose (Euthanyle, 400 mg/mL, 30 mL i.v., Brower, Buenos Aires, Argentina), and then full necropsy was performed. Samples taken for analysis included faeces, ileocaecal lymph node and fragments of ileum. Faecal samples from two bovines infected with MAP were obtained from a herd with endemic paratuberculosis. After proper decontamination, five drops of faecal samples were inoculated onto each of four Löwenstein Jensen (LJ; home made) slant media supplemented with 2 mg/L of mycobactin J (Allied Monitor, Inc., Fayette, Missouri, USA), without sodium pyruvate, and onto another four Herrold egg-yolk media (HEYM) with 2 mg/L of mycobactin J and 0.4% of sodium pyruvate (Whipple *et al.*, 1992). Tubes were incubated at 37 °C for 52 weeks and the growth was monitored by naked eye, stereomicroscope and microscopic examination (De Juan *et al.*, 2006; Whittington *et al.*, 1999). Putative MAP colonies grown on solid medium from bovine and ovine isolates were subjected to PCR as described by Collins *et al.* (1993). IS*1311* PCR/REA: amplification of the IS*1311* sequence (primers in Table 1) digested with *Hinf*I and *Mse*I endonucleases

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Table 1 - Oligonucleotide primers used for polymerase chain reaction (PCR) amplification of mycobacterial DNA.

Test	Name	Sequence 5'-3'	Target	Source
PCR	Dir C	GATCGGAACGTCGGCTGGTCAGG	IS*900*	Collins *et al*., 1993
PCR	Rev C	GATCGCCTTGCTCATCGCTGCCG		
Multiplex PCR	DMC529	TTGACAACGTCATTGAGAATCC	IS*900* sheep	Collins *et al*., 2002
Multiplex PCR	DMC531	TCTTATCGGACTTCTTCTGGC	IS*900* cattle	
Multiplex PCR	DMC533	CGGATTGACCTGCGTTTCAC	IS*900* sheep-cattle	
IS1311 PCR/REA	M56	GCG TGA GGC TCT GTG GTG AA	IS*1311*	Marsh *et al*., 1999
IS1311 PCR/REA	M119	ATG ACG ACC GCT TGG GAG AC	IS*1311*	

was carried out as described by Marsh *et al.* (1999). Multiplex PCR: DNA from ovine and bovine samples was extracted according to Van Soolingen *et al.* (1991), and the PCR assay was performed as described by Collins *et al.* (2002); primer DMC529 had a concentration two times higher than primers DMC531 and DMC533 (primers in Table 1). The products were resolved by electrophoresis on a 2% agarose gel with 5% ethidium bromide and visualized in a Gel Doc XR documentation system (Bio-Rad, Hercules, CA, USA). RFLP: DNA was extracted as described previously (Van Soolingen *et al.*, 1991). RFLP analysis was performed as described by Van Embden *et al.* (1993). RFLP profiles were analysed as described by Pavlik *et al.* (1999) and Green *et al.* (1989). At histopathology, large numbers of macrophages were seen throughout the mucosa and lamina propria with numerous acid-fast bacilli (AFB),

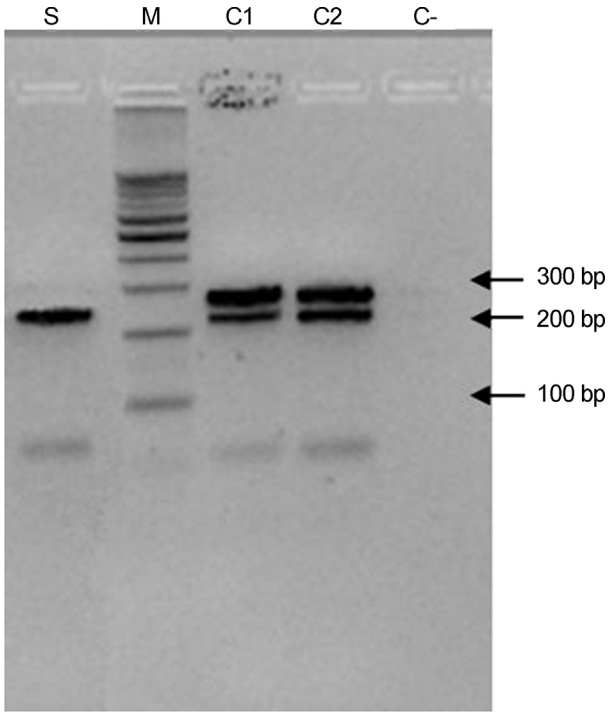

Figure 1 - IS*1311*-PCR/REA PCR-Restriction endonuclease analysis was performed with *Hinf*I and *Mse*I endonucleases to differentiate between bovine and ovine MAP isolates. Lane 1: S sheep strain; lane 2: M molecular marker (100 bp DNA ladder, Invitrogen); lane 3: C1 cattle strain; lane 4: C2 cattle strain; lane 5: C - negative control.

and lymphocytes were scattered throughout the lamina propria and mucosa, as previously reported (Perez *et al.*, 1996). A valid test was confirmed by the presence of more than one precipitin line indicating a strong positive reaction. Ovine samples were culture-positive on the LJ media with mycobactin J after six months of incubation. Colonies of the ovine strain were very small and with no pigmentation. No growth was observed in HEYM with mycobactin J. Positive culture for the bovine strains was observed after three months of incubation on Herrold's egg yolk slants. Colonies from cultures of ovine ileum and cattle faeces suspected to be MAP based on their morphology, slow growth rate and staining of acid-fast rods were confirmed by PCR on the base of the presence of IS*900*. Restriction endonuclease BstEII analysis showed two different profiles representing the S and C strains (Figure 1). These profiles match with those seen in earlier studies (Collins *et al.*, 1990; Pavlik *et al.*, 1999). Both IS*1311*-PCR/REA and DMC-PCR classified the ovine strain as S type and the bovine isolates as C type. Examination of ileal Ziehl-Neelsen (ZN) smears effectively demonstrated abundant AFB. Histopathological findings showed diffuse inflammatory infiltrate with large numbers of macrophages. This was also reflected in the ZN sections with high numbers of AFB located intracellulary, typical of the multibacillary (lepromatous) form of paratuberculosis. These observations correlate with clinical signs and the presence of a high mycobacterial load in intestinal lesions, Juste *et al.* (1991) and Whittington *et al.* (1999) showed that MAP culture from sheep is possible in the case of appropriate media usage. In order to improve culture of the sheep strain isolate, LJ medium supplemented with mycobactin J and without sodium pyruvate and HEYM with sodium pyruvate and mycobactin J were used. LJ was found to support the growth of the ovine strain after approximately six months of incubation, with very small colonies observed. In HEYM, which is frequently used to isolate MAP from cattle, no growth was observed for the sheep strain after one year of incubation. The growth requirements observed in this study are in agreement with reports (De Juan *et al.*, 2006) demonstrating different phenotypic characteristics of the S and C strains of MAP. MAP was confirmed by PCR amplification of the IS*900* element. Using RFLP analysis, IS*1311*-

PCR/REA and a multiplex DMC-PCR, the ovine strain was demonstrated to belong to the sheep S group and bovine strains to the cattle C group, confirming for the first time that both of these MAP strains are present in Argentina.

Acknowledgments

We would like to thank Dr Douglas Begg (from the Faculty of Veterinary Science, University of Sydney, Australia) for his critical reading of the manuscript and helpful suggestions and Petra Svastova (Veterinary Research Institute, Brno, Czech Republic) for technical support. The work was supported by the Ministry of Agriculture (No. MZe0002716202, QH81065) and by the Ministry of Education, Youth and Sports (AdmireVet, CZ 1.05/2.1.00/01.0006; ED0006/01/01.) of the Czech Republic.

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