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Paratuberculosis in sheep: Histochemical, immunohistochemical and *in situ* hybridization evidence of *in utero* and milk transmission

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ABSTRACT

To investigate *in utero* and milk transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), tissues from thirteen pregnant sheep, naturally infected and serologically positive to MAP, were examined by means of histochemistry, immunohistochemistry and *in situ* hybridization. Soon after parturition, ewes were euthanized and tissues samples were collected and prepared. The offspring (18 lambs) were divided into three groups to investigate different routes of MAP transmission. Lambs were sacrificed at three months old and the tissue samples collected, formalin-fixed and paraffin embedded. Hematoxylin and eosin and Ziehl–Neelsen staining methods were performed on fixed tissues for general examination and for detection of acid-fast bacteria. Additionally, immunohistochemical and *in situ* hybridization techniques were used to detect MAP antigen and MAP DNA respectively. This study of a flock of MAP-infected sheep indicates both *in utero* and milk transmission of MAP from dams to their offspring. Importantly, this study detected the presence of MAP in the mammary gland and mammary lymph nodes of adult ewes therefore indicating a significant route for the potential exposure to humans from this bacterial infection.

1. Introduction

Paratuberculosis is a chronic disease with an increasing and emerging clinical importance for animal and public health. The etiological agent involved is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease has been described primarily in both domestic and wild ruminants (Williams et al., 1983; De Lisle et al., 2003; Huntley et al., 2005; Thompson et al., 2007; Witte et al., 2009) and is distributed mainly in countries with advanced animal husbandry techniques where it has had significant economic and welfare impacts on meat and milk production (Harris and Barletta, 2001). The disease is characterized by weight loss, dehydration and profuse diarrhea in adult cattle and sheep between two to five years old. Diarrhea is not a constant finding in small ruminants (Carrigan and Seaman, 1990; Munjal et al., 2005; Robbe-Austerman, 2011; Khare et al., 2009). Postmortem findings are characterized by a chronic proliferative and histiocytic enteritis localized especially to the ileum with regional lymphadenopathy (Burrells et al., 1998; Khare et al., 2009). The economic losses first sparked scientific interest, while recently focus has shifted towards a possible link between MAP and human Crohn's disease (Hermon-Taylor et al., 2000; Hermon-Taylor, 2001; Sechi et al., 2001, 2004, 2005; Liverani et al., 2014). In support of this hypothesis, Sechi et al. (2001) examined biopsies from patients with Crohn's disease and demonstrated MAP in more than 70% of cases and numerous studies isolated the pathogen in raw milk from infected bovines (Giese and Ahrens, 2000; Ayele et al., 2005). In addition, pasteurization reduces MAP numbers in milk without complete elimination (Sung and Collins, 1998; Gao et al., 2002; Cavirani et al., 2003). Worryingly, MAP was detected by PCR in powdered milk used for feeding infants from seven European countries. Hruska et al. (2005) and Lambeth et al. (2004) showed colostrum to be the main transmission route to animal progeny due to high numbers of inflammatory cells present. MAP is excreted in the feces and may persist in the environment for years. Clinically infected sheep excrete large numbers of bacteria and therefore a single sheep may be responsible for a whole flock infection. For this reason the fecal-oral route is considered the main mode of transmission among animals due to food contaminated with feces (Sweeney, 1996). It has been proposed that animals are infected at less than 6 months old, with highest susceptibility at less than 30 days old, before the immune system is fully developed and has a prolonged incubation period averaging 2 years before the

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onset of clinical signs (Clarke, 1997). Whittington and Windsor (2009) have previously demonstrated *in utero* transmission in bovines. With respect to lambs, transmission of bacteria can occur *via* suckling contaminated teats or through the ingestion of infected milk. Limited evidence of *in utero* and milk transmission in sheep was obtained by Lambeth et al. (2004), in which gross, histological and culture findings indicated these transmission routes. This study aims to expand and further investigate this hypothesis through the course of a natural infection and using immunohistochemistry and *in situ* hybridization techniques.

2. Materials and methods

2.1. Selection of ewes

Thirteen, 2–3 year old Sardinian ewes, serologically positive to MAP (PTB-indirect ELISA, Istituto Zooprofilattico Sperimentale Lazio and Toscana, section of Viterbo, Italy) with no clinical signs of paratuberculosis were included in this study. Positive subjects were identified from a flock with a high prevalence of MAP infection. Five sheep from a flock that tested negative for MAP (ELISA, fecal culture and PCR on feces) were the negative controls. Negative controls showed no gross lesions at necropsy and were negative for MAP with specific *in situ* hybridization.

2.2. Treatment

Reproductive cycles were synchronized using flugestone acetate intravaginal sponges (Chronogest CR, MSD Animal Health, Milton Keynes, UK) and the ewes were bred with one ELISA-MAP-negative ram from the control flock one week post-removal of sponges. Ewe pregnancies were monitored *via* monthly ultrasound scanning and lambed under constant supervision in separate pens. Lambs were snatched from their mothers less than 5 min after birth and were not allowed access to dams after parturition. These lambs were cleaned and dried away from the dams to reduce contamination from maternal feces in the peri-parturant period.

2.3. Selection of lambs

Eighteen lambs were included in the study (13 lambs from positive ewes and 5 from the negative flock). The lambs were divided into three groups, housed in separate buildings (after thorough steam cleaning and disinfection), away from adult sheep to prevent direct contact or cross-contamination of feces. Disinfectant footbaths, hand washes, separate personal protective clothing and feeding devices were used between houses to decrease the risk of accidental contamination by personnel and fomites. Lamb groups were:

Group A) *in utero* transmission - 5 lambs, born from positive ewes and fed with a commercial freeze dried colostrum/milk replacer product (Farm-O-San-Colostrum and Farmilk Agnelli, Nutreco S.P.A., Italy).

Group B) milk transmission - 5 lambs, born from the negative control ewes and fed with colostrum/milk from the infected ewes after teat washing and disinfection followed by manual milking, hand washing and changing gloves between ewes.

Group C) *in utero* and milk transmission - 8 lambs, born from positive ewes and fed with colostrum/milk from their own mothers milked in the same way as described for group B.

2.4. PCR of feed

Despite the commercial colostrum/milk company advertising the product as MAP-free PCR was preformed to confirm this. Twenty mg of dry milk was diluted in 200 μl of MAP-free distilled water. DNA was isolated by DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to manufacturer's instructions. Adult feed (hay and concentrate pellets) was also tested by means of PCR to rule out a MAP contamination. Feed samples were homogenized and DNA was extracted using commercially available DNA extraction kits (ChargeSwitch gDNA Plant Kit from Invitrogen).

2.5. Sample collection

Monthly blood samples from each animal were analysed by ELISA to detect antibody titers against MAP. Ewes and lambs were checked daily for clinical signs (loss of weight, diarrhea) suggestive of MAP infection. The placentas were collected post parturition. When the lambs reached three months old, ewes and lambs were euthanized (intravascular injection of 20 ml of pentobarbital sodium 20% followed by intravascular administration of 15 ml of a cocktail of embutramide, mebenzonium iodide and tetracaine chlorhydrate - Tanax®, MSD Animal Health, Italy) and underwent a postmortem examination. Samples of ileum, mesenteric lymph nodes, mammary gland, mammary lymph nodes and uterus were collected from ewes and samples of ileum, mesenteric lymph nodes, thymus, pharyngeal lymph nodes and tonsils were collected from each lamb. Disinfection of instruments was performed when collecting different tissue samples to prevent cross-contamination.

2.6. Histopathology and histochemistry

Tissue samples were fixed in 10%, buffered formalin, dehydrated, and paraffin wax embedded. Serial sections (4.0 μm) were stained with hematoxylin-eosin (HE) for general examination and with a commercial Ziehl-Neelsen (ZN) stain kit (product code: 01020, DiaPath S.P.A., Italy) to detect acid-fast bacteria.

2.7. Immunohistochemistry

Immunohistochemistry (IHC) was performed using the streptavidin-biotin technique (Rocca et al., 2010) using a polyclonal antibody raised in rabbit (Dako, S.R.L., Italy), directed against *Mycobacterium bovis* (1:1000). Sections were deparaffinized and endogenous peroxidases inhibited with 0.3% hydrogen peroxide in methanol for 30 min while antigen retrieval was achieved using 0.05% protease XIV at 37 °C for 5 min. Sections were incubated at room temperature for 1 h with the primary antibody, rinsed and incubated with the secondary antibody at room temperature for 45 min. Sections were incubated with the streptavidin-biotin-peroxidase complex (Dako, S.R.L., Italy) at room temperature for 45 min with diaminobenzidine (DAB) for 10 s and blocked with sterile water. Sections were counterstained with hematoxylin, dehydrated and mounted. To validate the polyclonal antibody cross-reaction between *Mycobacterium bovis* and MAP, samples of ileum from a MAP-infected sheep (ELISA and PCR confirmed) with clinical signs were used. These positive control tissues demonstrated high positive immuno-labelling in the cytoplasm of macrophages infiltrating the ileal mucosa. The negative controls used were ileum samples obtained from the negative control sheep.

2.8. In situ hybridization

In situ hybridization (ISH) was applied to samples of uterus and placenta from the adult ewes and on ileum and mesenteric lymph nodes from lambs, using biotinylated DNA probes, specific for the 254-bp MAP F57 protein codifying gene (Vansnick et al., 2004). The F57 sequence was selected due to its high specificity for the MAP genome (Tasara and Stephan, 2005). Sections were deparaffinized, rehydrated, digested with pepsin (0.8% in HCl 0.2 N) for 30 min at 37 °C and washed in PBS and sterile water.