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

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**Abstract**

To demonstrate *in utero* and milk transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a group of pregnant Sardinian sheep (13), 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 demonstrate different routes of MAP transmission. Lambs were sacrificed at three months old and their 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 completed to detect MAP antigen and MAP DNA respectively. This study of a single 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 proposing a significant route for the potential exposure to humans from this bacterial infection.

**Keywords:** Sheep, paratuberculosis, *in utero*, milk transmission, immunohistochemistry, *in situ* hybridization.

**Introduction**

Paratuberculosis or Johne’s disease 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), belonging to *Mycobacteriaceae* family. The disease has been described primarily in both domestic and wild ruminants (De Liesle *et al.*, 2003; Huntley *et al.*, 2005; Thompson *et al.*, 2007; Williams *et al.*, 1983; Witte *et al.*, 2009) and is mainly spread in Western countries with advanced animal husbandry techniques where it has had significant negative economical and welfare impacts on meat and milk production (Harris and Barletta, 2001). The disease is characterized by weight loss, dehydration and profuse diarrhea that is particularly notable in adult cattle and sheep between two to five years old. Although diarrhea is not a constant finding in small ruminants, when compared to cattle, if it does occur it usually subsides within several weeks of symptom onset or is intermittent while weight loss remains the predominant feature (Carrigan and Seaman, 1990; Khare *et al.*, 2009; Munjal *et al.*, 2005; Robbe-Austerman, 2011). Decreased fertility and reduction in milk production among breeding and lactating females are common findings along with other symptoms such as recurrent fever and submandibular edema. Post mortem findings are characterized by chronic proliferative and histiocytic enteritis localized especially to the ileum with regional lymphadenopathy(Burrels *et al.*, 1998; Khare *et al.*, 2009). Scientific interest for paratuberculosis was first generated as a result of the economic losses in the cattle industry, while in recent years focus has been directed towards a possible link between MAP and human Crohn’s disease (Hermon-Taylor *et al.*, 2000, 2001; Liverani *et al*., 2014; Sechi *et al.*, 2001, 2004, 2005). In support of this hypothesis, Sechi *et al.* (2001) in a study 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 (Ayele *et al.*, 2005, Giese and Ahrens, 2000). In addition, pasteurization has been demonstrated by other authors to reduced MAP numbers in milk without complete elimination (Cavirani *et al.*, 2003; Gao *et al.*, 2002; Sung and Collins, 1998). Worryingly, in a study performed on powdered milk used for infant feeding from seven European countries, Hruska *et al.* (2005) demonstrated the presence of MAP by PCR while 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 eliminated (or shed) in the feces of ruminants grazing at pasture and may persist in the environment for years. Clinically infected sheep expel enormous amounts 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 on pasture due to food contaminated with feces(Sweeney, 1996). The main hypothesized pathogenesis is animals are infected at less than 6 months old, with the susceptibility to infection being highest at less than 30 days old, by the fecal-oral route before the immune system is fully developed and a prolonged incubation period averaging 2 years or more before the onset of clinical signs (Clarke, 1997). Whittington and Windsor (2009) have previously demonstrated *in utero* transmission in bovines. With respect to lambs, the passage of the bacterium can occur through suckling from contaminated teats or through the ingestion of infected milk. There is limited scientific evidence regarding *in utero* and milk transmission in sheep performed by Lambeth *et al.* (2004), in which gross, histological and culture findings preliminary suggested these modes of transmission. We have therefore attempted in this study to expand and further demonstrate this hypothesis through the course of a natural infection and using immunohistochemistry and *in situ* hybridization techniques.

**Materials and Methods**

A group of 13, 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 single flock of sheep with high prevalence for MAP infection after serological screenings were performed at 4 farms in the Lazio region, Italy. A group of 5 sheep from a single flock from the province of Pisa, Italy that tested negative for MAP (ELISA, fecal culture and PCR on faeces) represented the negative control group. This last group showed no gross evidence of paratuberculosis at necropsy and no intestinal positivity for MAP with specific *in situ* hybridization. All ewes from the experimental groups (controls and MAP-positive animals) were synchronized by use of intravaginal sponges containing flugestone acetate (Chronogest CR, MSD Animal Health, Milton Keynes, UK) , to shorten the lambing period, and bred with one ELISA-MAP-negative ram from the control flock one week after the removal of sponges. Monthly ultrasound scanning was performed on the gravid ewes to monitor pregnancy. The ewes lambed under constant supervision in separate pens within their enclosure. Lambs were snatched from their mothers less than 5 minutes after birth and ewes were not allowed to touch the lambs after parturition. These lambs were cleaned and dried away from the dams to try and reduce any contamination that may have occurred from maternal feces during the peri-parturant period. After parturition, 18 lambs were available and included in the study (13 lambs from positive sheep and 5 from the negative flock). The lambs were divided into three groups, kept in separate buildings (after thorough steam cleaning and disinfection) and housed separate from the adult sheep enclosure to prevent the possibility of direct contact or cross-contamination of feces. Additionally, using disinfectant footbaths, hand washes, separate personal protective clothing and feeding devices between houses decreased the risks of accidental contamination by personnel and fomites.

Lamb groups are as follows:

Group A) *in utero* transmission - 5 lambs, born from positive ewes and fed with a commercial freeze dried colostrum/milk replacer.

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

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

For the Group A lambs which required feeding with a milk replacer, a commercial freeze-dried colostrum/milk product (Farm-O-San-Colostrum and Farmilk Agnelli, Nutreco S.P.A. Italy) was used. Despite the commercial colostrum/milk company advertising the product as MAP-free it was PCR analysed to exclude the presence of MAP contamination. A total 20 mg of dry milk samples were 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 cross contamination. From the feed, a homogenized sample was prepared in a blender. DNA was extracted from homogenized material using commercially available DNA extraction kits (ChargeSwith gDNA Plant kit from Invitrogen).

Blood samples were collected monthly from all subjects (ewes and lambs) and analysed (ELISA) to verify the persistence of antibody titers against MAP. Ewes and lambs were checked daily for possible clinical signs (loss of weight, diarrhea) suggestive of MAP infection. The placentas from all ewes were collected post parturition. At the end of the study period, when the lambs reached three months old, ewes and lambs were euthanized (intravascular injection of 20ml of Pentobarbital sodium 20% followed by intravascular administration of 15ml of a cocktail of embutramide, mebenzonium iodide and tetracaine chlorhydrate-Tanax®, MSD Animal Health, Italy) and underwent a post mortem 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 preformed when collecting different tissue samples to prevent cross-contamination with MAP between the samples that could have potentially led to dubious PCR results.

All tissue samples, were fixed in 10%, buffered formalin, dehydrated, and paraffin embedded. Four µm thick serial sections were stained with Hematoxylin-Eosin (HE) for general examination and with commercial Ziehl-Neelsen (ZN) stain kit (product code: 01020, DiaPath S.P.A. Italy) to detect acid-fast bacteria.

Immunohistochemistry (IHC) was performed on the sections using the streptavidin-biotin technique (Rocca *et al.*, 2010) by means of a polyclonal antibody raised in rabbit (Dako, S.R.L. Italy), directed against *Mycobacterium bovis* at the dilution of 1:1000. Briefly, sections were deparaffinized and endogenous peroxidases were inhibited with 0.3% hydrogen peroxide in methanol for 30 minutes while antigen retrieval was achieved using 0.05% protease XIV at 37°C for 5 minutes. Sections were then incubated at room temperature for 1 hour with the primary antibody, rinsed and incubated with the secondary antibody at room temperature for 45 minutes and subsequently with the streptavidin-biotin-peroxidase complex (Dako, S.R.L. Italy) at room temperature for 45 minutes. The reaction was created by using diaminobenzidine (DAB) for 10 seconds and blocked by sterile water. Sections were counterstained with hematoxylin, dehydrated and mounted permanently. To validate the polyclonal antibody cross-reaction between *Mycobacterium bovis* and MAP, samples of ileum, from a MAP-infected sheep (ELISA and PCR confirmed) of the same flock of Sardinian ewes with evident 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 from Pisa. *In situ* hybridization (ISH) was performed on the uterus and placenta from the adult ewes, and on ileum and mesenteric lymph nodes from lambs using biotinilated 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 as reported by Tasara *et al.* (2005). Sections were deparaffinised, rehydrated, digested with pepsin (0.8% in HCl 0,2N) for 30 minutes at 37°C and washed in PBS and sterile water.

Subsequently, tissues were dehydrated, and put into a moist chamber at 38°C for two hours, with a pre-hybridization solution. This solution was then replaced with biotinilated probes F57A-5'-GGTCGCGTCATTCAGAATC-3' F57P2-5'-AGTGGGAGGCGTACCAGGGTC-3', and slides were submitted to denaturation at 98°C for 8 minutes, in a thermal cycler (Hybaid Omnislides, Thermo Scientific, Waltham, MA, USA) and then incubated overnight at 38°C in a moist chamber. The following day, stringency washes were performed at decreasing SSPE concentrations (2x, 1x, 0.5x, 0.1x at 50°C for 30 minutes). The solution was eliminated through PBS washing, endogenous peroxidases were blocked and then samples were incubated with extravidin. The reaction was revealed using DAB for 10 seconds. After washing in sterile water, slides were mounted with aqueous medium, examined with a light microscope, and images captured. To observe the fluorescence on the same slides, the coverslips were removed, the sections washed in double distilled water and incubated for 2 hours in the dark at room temperature with anti-avidin FITC-antibody. Nuclei were counterstained with Hoechst for 5 minutes, washed with sterile water, and mounted with an aqueous medium. They were then examined with a fluorescence microscope and images were captured. Intestine samples from a MAP-positive sheep, confirmed by ISH, PCR and culture were used as positive controls. Ileum samples from a sheep of the control group were used as negative controls.

**Results**

Adult MAP-infected Ewes

*Serology and gross findings*

Serology performed on adult MAP-infected ewes monthly, consistently showed positive results in the absence of clinical manifestation of paratuberculosis. Necropsies, performed on all adult sheep (13), detected lesions of varying degrees of severity, especially in samples localized to the ileum and its draining lymph nodes. In 3 out of 13 ewes the lesions extended to the entire small intestine. In particular, lymphatic vessels and mucosal proliferation were evident on the surface of the small intestine. Lymph nodes appeared enlarged and edematous on the cut surface while other organs did not show any macroscopic lesions suggestive of paratuberculosis.

*Histopathology/histochemistry/immunohistochemistry/in-situ hybridization*

*Terminal ileum*

In our study, two distinct forms of microscopic lesions of the ileum were recognized similar to those previously described by Clarke and Little (1996) with a high or a low degree of mycobacterial colonization (“multibacillary” or “paucibacillary”). In the multibacillary form, lesions resembled those of lepromatous leprosy and the ileum was infiltrated by sheets of closely packed macrophages. This infiltrate distended the lamina propria often spreading into the submucosa with a mild to moderate diffuse lymphocytic infiltration admixed with macrophages. The cellular infiltrate surrounded and compressed the intestinal crypts. There was also moderate to severe atrophy and fusion of villi, resulting in an attenuated mucosal surface. In the “paucibacillary” form lesions showed a different histological pattern characterised by multifocal granulomas resembling tubercles. In this form, the lamina propria and the submucosa was infiltrated predominantly by small lymphocytes. Multifocal aggregates of large histiocytic cells were consistently present but usually organised into small granulomas. These granulomas were never encapsulated and showed no evidence of necrosis or calcification. Multinucleated giant cells (Langhan’s type) were occasionally observed. Cellular infiltrate extended into the submucosa and reflected the predominance of lymphocytes. All macrophages distributed in small groups showed fewer intracytoplasmic acid-fast organisms than in the multibacillary form by means of ZN stain.

Five out of 13 subjects exhibited lepromatous lesions with ZN staining detecting a multibacillary infection characterized by the presence of epithelioid macrophages with cytoplasm-filled acid-fast bacteria (Fig. 1a). In the other 8 out of 13 subjects, a tuberculoid pattern of enteritis was detected and a paucibacillary infection was observed (Fig 1b). This was characterized by scattered acid-fast positive bacteria often forming clusters within the cytoplasm of macrophages which were dispersed among numerous lymphocytes and plasma cells that constituted the majority of the inflammatory infiltrate. Histochemical findings, obtained through ZN staining, were confirmed by IHC showing the same positive immune-labelling within the cytoplasm of macrophages (Fig. 1b inset).

*Mesenteric lymph nodes*

The mesenteric lymph nodes in almost all adult infected ewes were hyperplastic with an excessive expansion of secondary follicles. Similar to the enteric lesions, two main histopathological reactive patterns were detected. The first one was associated with lepromatous enteric lesions, characterized by epithelioid cells within the medullar and sub-cortical sinuses; and the second one was associated with tuberculoid enteric lesions with fewer epithelioid cells and scattered multinucleated giant cells. All 13 samples tested positive with ZN and with the polyclonal antibody within the macrophage cytoplasm distributed mainly near the cortical areas. ISH was not performed on adult ewe mesenteric lymph node.

*Uterus*

Morphologically the uterus appeared normal in all animals except in 2 subjects (ewe No. 4 and 9) also positive to ZN, IHC and ISH for MAP, in which a diffuse chronic endometritis was present. This was characterized by diffuse epithelioid infiltrate and dysplasia of the endometrial glands caused by compression from surrounding lympho-plasmacytic infiltrates.

In nine subjects, ZN staining (Fig. c) confirmed by IHC (Fig. 1d) demonstrated the presence of acid-fast bacteria within epithelioid cells. These positive results were confirmed by ISH on the same sections (Fig. 1d inset).

*Mammary gland*

No specific lesions were evident in the mammary gland parenchyma with HE staining. ZN could demonstrate the presence of acid-fast bacteria in one of the 13 subjects tested, while immunohistochemistry was able to detect MAP antigen in 8 cases (Fig. 1e). ISH was not performed on the mammary gland.

*Mammary lymph nodes*

Contrary to what was observed in mammary gland parenchyma, ZN stain demonstrated acid-fast bacteria in almost all samples (10/13) of mammary lymph nodes and these results were confirmed by IHC performed on the same sections (Fig. 1f). Even in these tissues, positive findings were localized to macrophage cytoplasm. ISH was not performed on mammary lymph nodes.

*Placenta*

ZN stain did not demonstrate MAP in any of the placental sections examined, however IHC was able to detect bacterial antigen in 10 animals within the mucosal cotyledonal cells and within the cytoplasm of macrophages (Fig. 2g). In addition, further placental localizations of MAP were obtained by ISH, confirming the positivity obtained by the polyclonal antibody (Fig. 2h).

All the results presented in this section are summarized in Table 1.

Lambs

*Serology and gross findings*

One lamb from group C died after 2 months secondary to a bacterial pneumonia, clearly not related to paratuberculosis. Despite this, organs from the lamb were collected, submitted and included for MAP investigation. All lambs, in the study period, consistently showed negative results for the presence of serum antibodies (ELISA), except for one subject from group C that seroconverted without clinical manifestations. At necropsy, all subjects from the 3 groups, showed moderate intestinal lesions characterized by pleating of the mucosal surface with a rare reddish appearance accompanied in all cases by a mild to moderate increase in size of mesenteric lymph nodes.

*Histopathology/histochemistry/immunohistochemistry/in situ hybridization*

All intestinal samples from lambs belonging to the three different groups showed moderate chronic active diffuse enteritis mostly associated with a lymphoplasmacellular infiltrate. Lymph nodal lesions were related to a hyperplastic lymphadenitis without a clear division between follicular structures that, in some cases, appeared fused. ZN stain and IHC detected MAP in the ileum of 2 out of 5 and 3 out of 5 lambs from group A respectively, while mesenteric lymph node samples from the same group tested positive with both ZN and IHC in 3 out of 5 subjects (Table 2). Ileum samples from group B were detected positive for ZN stain in 2 out of 5 subjects and in 4 out of 5 subjects using IHC (Table 2). Mesenteric lymph nodes of the same group tested positive with ZN stain in 3 out of 5 animals while IHC detected the presence of mycobacteria antigens in 4 out of 5 animals (Table 2).

Ileum samples and mesenteric lymph nodes of the last group (group C) tested positive both for ZN and IHC in 5 out of 8 lambs and 6 out of 8 lambs respectively (Fig. 2i). The positive results in this last group were furthermore higher than the other groups with regard to the intensity of staining of the cytoplasm of macrophages with both IHC and ISH techniques. IHC results were confirmed in all three groups both in the ileum and mesenteric lymph nodes by ISH (Fig. 2l).

**Discussion**

This study was performed to demonstrate possible antenatal, perinatal and postnatal routes of transmission of *Mycobacterium avium* subsp. *paratuberculosis* in ovine species. Specifically, *in utero* and milk transmission routes were evaluated through the use of pregnant ewes sourced from a single MAP-positive flock. To our knowledge this is the first study that revealed this occurrence in sheep with no clinical signs of paratuberculosis by means of immunohistochemical and hybridization techniques. Despite ewes displaying no clinical signs for Johne’s disease and consistently showing ELISA positivity, there were evident gross lesions of varying severity and in 3 cases spanning the length of the small intestine which goes against the findings of Gillian *et al* (2010) which states that asymptomatic animals have no gross or histological signs. It is possible that these animals may have developed an immune response to suppress the onset of clinical signs as suggested by Pérez *et al* (1996)and therefore have some degree of gross lesions despite not having the clinical presentation of the disease. Gross lesions (related to MAP) in the adult ewes were restricted to the small intestine and lymph nodes.

Our results suggest both *in utero* and milk transmission routes within this species can occur with IHC and ISH techniques displaying a higher sensitivity to detecting MAP positive cells than traditional ZN staining.

The increased sensitivity of IHC and ISH is exhibited by the results in the placenta and mammary gland where by the tissues were negative to MAP using ZN staining methods but positive using IHC and as for the placenta ISH. In 9 out of the 13 ewes the uterus and placenta were positive for MAP on IHC and ISH supporting the hypothesis of potential *in utero* transmission route. Additionally, ZN staining and IHC (confirmed by means of ISH) revealed positive results for MAP in intestine and mesenteric lymph nodes of lambs from group A (*in utero* infection), born from MAP positive mothers but fed with colostrum/milk replacer PCR negative for MAP.

The detection of MAP in the ovine mammary gland and especially in the ovine mammary lymph nodes reinforces the potential for exposure to humans by MAP. Recently, in regard to this, MAP RNA has been identified by means of liquid phase RT-PCR in raw ovine milk from naturally infected animals and also in commercial bovine pasteurized and UHT milk (Cubeddu *et al.*, 2008). Lambeth *et al.* (2004) identified MAP DNA by means of PCR in samples of ovine milk from γ-interferon positive tested sheep. The authors also hypothesized colostrum as an important route for perinatal infection for two main reasons: firstly colostrum contains an inherently high number of macrophages, the cells in which mycobacteria reside, and secondly because the colostrum is consumed by lambs within the time period in which intestinal M-cells are more receptive to antigens (Kurade *et al.*, 2004; Ponnusamy *et al*., 2013). This assumption is supported in our study with data from group B (milk transmission) lambs that showed positive results to MAP in the intestine and mesenteric lymph nodes. Another interesting finding is the evidence of a possible combined transmission route (i.e. *in utero* and via milk) that occurred in group C where lambs were snatched immediately after birth to prevent contact with feces and other contaminated products from their mothers. The animals in group C appeared to be the most affected as demonstrated, in at least one subject, by seroconversion for MAP (detected by ELISA) and by displaying the highest intensity of positive staining in the macrophage cytoplasm among the three lamb groups confirmed by ZN, IHC and ISH despite having slightly lower percentage positive animals than group B. From data of group C, an assumption for higher infectivity in lambs could be a result of infected dams as well as their feeding with infected colostrum/milk. These results follow the findings of McGregor *et al* (2012) and Delgado *et al* (2013) that showed a higher infective dose of MAP administered to young animals produced more severe lesions when they are adult animals. The relative increase in gross changes in the intestine of lambs compared to adult animals may be due to the earlier and more intense cell mediated immune response in infected adult ewes limiting the gross lesions (Delgado *et al*, 2011). In conclusion, although this study sample size is small and assumptions cannot be fully determined, this combined transmission route should be considered as a major contributor to the natural epidemiologic course of MAP in sheep.

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