



## DIAGNOSIS OF PARATUBERCULOSIS IN FRESH AND PARAFFIN EMBEDDED SAMPLES BY HISTOPATHOLOGY, PCR AND IMMUNOHISTOCHEMISTRY TECHNIQUES

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### Summary

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*Mycobacterium avium* subsp. *paratuberculosis* (Map) is the causative agent of paratuberculosis, also called Johne's disease. Affected ruminants show emaciation, decreased milk production, oedema, anaemia and infertility. The objective of the present study was to compare histopathology, immunohistochemistry (IHC) and IS900-based PCR techniques for diagnosis of ovine paratuberculosis in archival fresh and paraffin-embedded tissues. A total of 24 samples (11 fresh and 13 older than 1 year paraffin-embedded intestines) grossly suspected to ovine paratuberculosis were used. By histopathology, Ziehl-Neelsen, and IHC methods, 9/11 fresh and 5/13 paraffin-embedded tissues were positive for paratuberculosis. PCR detected 413 bp amplicons in all histopathologically positive fresh and none of paraffin-embedded samples. In the present study, it seems that using PCR assay on the old paraffin-embedded tissues in comparison with the fresh samples cannot be useful although a higher number of samples is necessary to make a reliable conclusion.

**Key words:** *Mycobacterium avium* subspecies *paratuberculosis*, paraffin-embedded tissue sample, paratuberculosis, PCR, sheep

### INTRODUCTION

Paratuberculosis, also called Johne's disease, is a chronic bacterial infection caused by the acid-fast bacillus bacteria *Mycobacterium avium* subspecies *paratuberculosis* (Map). It occurs primarily in domestic and wild ruminants but a variety

of non-ruminants species are also affected. Incubation period is long and young animals are more susceptible to infection, however, clinical signs of the disease may be noticed after some year (Manning & Collins, 2001; Jubb *et al.*, 2007). Gene-

rally, diagnosis of paratuberculosis is difficult particularly in early and subclinical stages (Sigurdardottir *et al.*, 1999; Manning & Collins, 2001). Among the various diagnostic tests, bacterial culture is considered as a highly specific and definitive test for paratuberculosis, but it is very time consuming (up to 12 weeks) (Clark *et al.*, 2008; Singh *et al.*, 2008). In the cases that lesions are not specific enough, definitive diagnosis depends on the isolation of the organisms from the intestinal tissues and associated lymph nodes (Englunda *et al.*, 2001; Ellingson *et al.*, 2005). In paraffin embedded tissues, cultural examination is not possible. In such cases, IS900-based PCR is suggested for detection of Map DNA and for more conclusive diagnosis of paratuberculosis in histologically inconclusive cases (Whittington *et al.*, 1999; Erume *et al.*, 2001; Sivakumar *et al.*, 2005). PCR based assays especially IS900 gene is a unique, sensitive and rapid method for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* in different clinical samples including faeces, blood, milk as well as fresh and fixed tissues (Manning & Collins, 2001, Clark *et al.*, 2008; Singh *et al.*, 2008). The PCR assay has been used for direct detection of Map genome from formalin-fixed paraffin-embedded tissue samples with paratuberculosis (Fiallo *et al.*, 1992; Plante *et al.*, 1996; Whittington *et al.*, 1999; Miller *et al.*, 2002).

The purpose of the present study was to compare histopathology, immunohistochemistry (IHC) and IS900-based PCR techniques for diagnosis of ovine paratuberculosis in archival fresh and paraffin-embedded samples. Using archival specimens is important for retrospective and epidemiological studies at a large scale.

## MATERIALS AND METHODS

### *Sampling*

In this study, a total of 24 intestinal samples including 11 fresh and 13 paraffin-embedded intestines older than 1 year that grossly suspected to ovine paratuberculosis were studied. Fresh samples obtained from referred sheep with clinical signs of paratuberculosis that were necropsied and grossly showed thickening of intestines. The 13 paraffin-embedded intestines were obtained from our archives at the Pathology Department.

### *Microscopic investigations*

Samples from various regions of the intestines and associated lymph nodes about 1 cm<sup>3</sup> in diameter were taken and preserved in 10% neutral buffered formalin, dehydrated through a graded alcohol series and embedded in paraffin blocks. Sections at 5 µm thicknesses were cut and routinely stained with haematoxylin-eosin (HE) as well as Ziehl-Neelsen (ZN) acid-fast method and studied microscopically. Also, the avidin-biotin complex peroxidase immunohistochemical technique (IHC) on the intestine samples was applied first with biotinylated rabbit polyclonal anti-*Mycobacterium paratuberculosis* antibody (DAKO, Denmark). All 24 samples were examined by ZN, IHC and PCR methods. Also, 10 apparently normal intestines were used as controls.

### *Polymerase chain reaction (PCR) analysis*

A PCR technique that amplified a 413 bp DNA fragment was conducted on fresh (n=11) and paraffin-embedded (n=13) tissue samples. For paraffin-embedded specimens, two 20 µm (25 mg) thicknesses were cut from each paraffin block and placed in a 1.5 mL tube. Paraffin was dissolved by adding 1 mL of xylene and

place on a shaker for 15 min. Subsequently, the tubes were centrifuged at 12,000×g for 5 min. For removing the xylene, supernatants were disposed without disturbing of the pellets. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Quality of extracted DNA was investigated by agarose gel electrophoresis and ethidium bromide staining. Primers were chosen to amplify the 5' region of IS900. The primer pair P90 (5'GAA GGG TGT TCG GGG CCG TCG CTT AGG) and P91 (5' GGC GTT GAG GTC GAT CGC CCA CGT GAC) spans a 413 bp region. The reaction was performed in a total volume of 25 µL containing 5 µL of the DNA sample as template, 1 µL of each of the primers P90 and P91, 10 mM (0.5 µL) of dNTP, 50 mM (2.5 µL) MgCl<sub>2</sub>, 2.5 µL PCR buffer, 1 U (0.2 µL) Taq DNA polymerase and 12.3 µL distilled water. Amplification was undertaken in thermal cycler (BioRad, USA) using the following conditions: one cycle of predenaturation at 94 °C for 3 min followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplified products were analysed in 2% agarose gels containing 10 µL ethidium bromide. DNA of the certainly positive case and sterilised distilled water were used as positive and negative controls, respectively. A sample was considered positive when the 413 bp DNA fragment of the IS900 sequence was obtained.

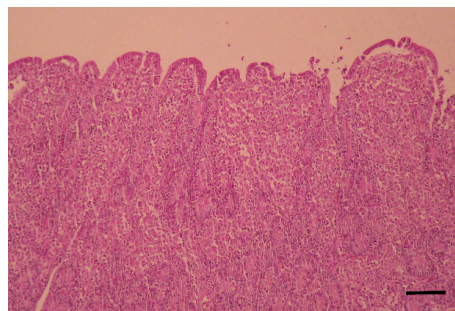
## RESULTS

### *Microscopic findings*

Fourteen (58.33%) out of the 24 examined sheep showed histopathological lesions associated with paratuberculosis. Of them,

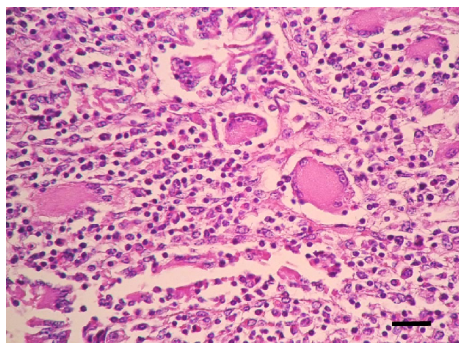
9 and 5 cases were fresh and paraffin-embedded tissue samples, respectively.

Histopathologically, the main lesions included diffuse granulomatous enteritis and focal to diffuse granulomatous lymphadenitis. Other inflammatory cells including variable numbers of lymphocytes, plasma cells as well as eosinophils were observed. In most cases, severe infiltration of epithelioid macrophages was detected in the tips of the villi, lamina propria and mucosa that caused thickening of mucosa as well as atrophy and obliteration of the crypts of Lieberkühn (Fig. 1). In some cases, other layers of the intestines especially the submucosa and serosa were infiltrated with inflammatory cells. In 3 cases, a lot of Langhans giant cells with variable numbers of nuclei were found in the intestinal mucosa (Fig. 2).

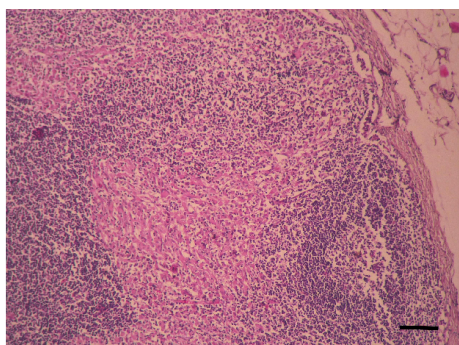


**Fig. 1.** Ileum. Severe infiltration of epithelioid macrophages in the intestinal mucosa especially in the tips of villi (HE, Bar=100 µm).

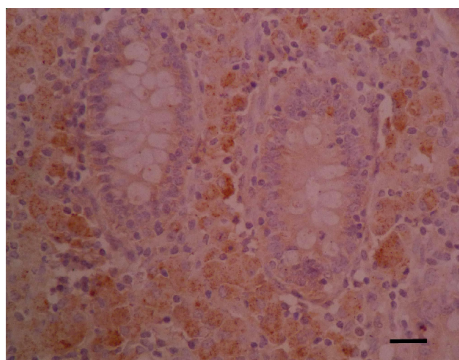
In the mesenteric lymph nodes, focal to diffuse inflammation composed of epithelioid macrophages as main inflammatory cells was present in the cortex, paracortex, subcapsular sinuses and rarely in the medulla (Fig. 3). Other inflammatory cells concluded Langhans giant cells, lymphocytes, plasma cells and rarely eosinophils. Caseous necrosis was seen in the mesenteric lymph nodes of one case.



**Fig. 2.** Ileum. Presence of Langhans giant cells in the lamina propria could be noticed (HE, Bar=25  $\mu$ m).



**Fig. 3.** Mesenteric lymph node. Diffuse granulomatous lymphadenitis (HE, Bar=100  $\mu$ m).

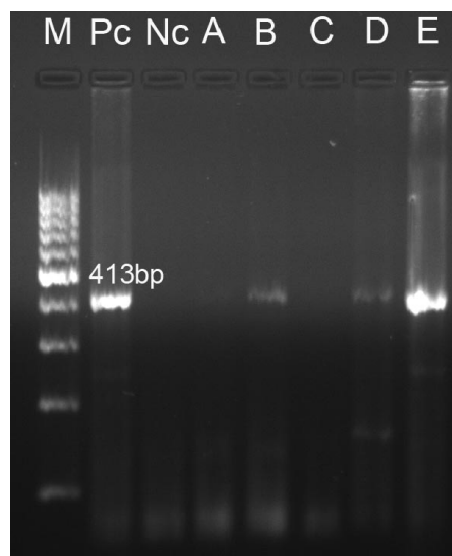


**Fig. 4.** Immunolabelling for MAP antigens exhibited a positive reaction in the cytoplasm of the epithelioid macrophages as brownish granules (Bar=25  $\mu$ m).

Positivity was confirmed as brownish granules by the IHC method (Fig. 4) and red acid-fast bacilli by ZN staining located in the cytoplasm of epithelioid macrophages. In all histopathologically positive tissue sections, mycobacteria were demonstrated by both IHC and ZN methods within macrophages.

#### *Detection of Map by PCR technique*

When PCR was applied on the DNA of the tissue samples, 9 out of 11 histopathologically positive cases also showed amplification of IS900 sequence of Map (Fig. 5). All of these positive cases were from the fresh samples. Amplicons of the expected size were not detected in the 5 paraffin-embedded samples that showed histopathological lesions of paratuberculosis (Table 1) and none of the 10 control intestines.



**Fig. 5.** Detection of IS900 sequence (413 bp) in tissue samples. M: markers; Pc: positive control; Nc: negative control; lanes B, D, E: positive amplification (413 bp); lanes A, C: negative amplification.

**Table 1.** Results of microscopic and PCR investigations in the fresh and paraffin-embedded tissue samples of sheep

| Source of samples | Number of samples | Histopathologically positive (%) | IHC positive (%) | PCR positive (%) |
|-------------------|-------------------|----------------------------------|------------------|------------------|
| Fresh             | 11                | 9 (81.81)                        | 9 (81.81)        | 9 (81.81)        |
| Paraffin embedded | 13                | 5 (38.46)                        | 5 (38.46)        | 0                |
| Total             | 24                | 14 (58.33)                       | 14 (58.33)       | 9 (37.5)         |
| Normal intestines | 10                | 0                                | 0                | 0                |

## DISCUSSION

In the present research, paratuberculosis was identified in 14 (fresh and paraffin-embedded) out of 24 intestinal samples by histopathological investigation, Ziehl-Neelsen (ZN) and IHC staining. IS900-based PCR detected 413 bp amplicons in all histopathologically positive fresh samples and none of older than 1 year paraffin-embedded tissues. The histopathological findings including diffuse granulomatous enteritis and lymphadenitis reported here were similar to those reported previous in sheep (Gonzalez *et al.*, 2005; Kheirandish *et al.*, 2008; Oryan *et al.*, 2008). Many studies have been conducted on histopathological characteristics of John's disease in different ruminant species including cattle, sheep and goats. The present study indicated close correlation of pathological examination with the ZN and IHC methods. It has been expressed that if appropriate tissue samples collection be performed, histopathological features will be suggested as a good parameter even more sensitive than bacteriological culture, immunological and molecular methods for diagnosis of paratuberculosis in sheep (Signurdardottir *et al.*, 1999; Manning & Collins, 2001). In our investigation, histopathological, ZN and IHC methods are considered as golden tests to

confirm the presence of *M. paratuberculosis*. For paratuberculosis, several histopathological classifications have been carried out in sheep based on the location of lesions, presence of the organisms and nature of the inflammatory cells (Clarke, 1997; Gonzalez *et al.*, 2005; Kheirandish *et al.*, 2008; Oryan *et al.*, 2008). These forms at histopathological level include diffuse multibacillary or lepromatous form accompanied by lesions consisting of numerous mycobacteria, epithelioid macrophages because of weak cell-mediated immune response. The other form is diffuse lymphocytic or paucibacillary (tuberculoid) form with lesions characterised by inflammatory infiltrate composed of lymphocytes, a few epithelioid macrophages, giant cells and scant numbers of mycobacteria due to strong cellular immune response. And, the last form namely diffuse mixed is described as diffuse mixed lesions in sheep that share features of both multibacillary and lymphocytic lesions. The obtained results revealed that diffuse multibacillary lesions were the commonest form confirmed in this study. This type has been previously suggested as the classic lesion of John's disease in sheep (Clarke, 1997; Kheirandish *et al.*, 2008). Diffuse lymphocytic lesions were observed in a few cases associated with low numbers of the acid fast bacteria in this

form. In accordance with others, we showed that caseous necrosis was an uncommon finding in the mediastinal lymph nodes (Gonzalez *et al.*, 2005; Kheirandish *et al.*, 2008).

By PCR analysis, only 9 cases performed a 413 bp fragment of DNA that belonged to fresh samples, however, all of paraffin-embedded tissue samples were negative.

The PCR assay has been used for detection of nucleic acid in DNA extracted from formalin-fixed paraffin embedded tissue sections in most mycobacterial infections of humans and animals including paratuberculosis (Fiallo *et al.*, 1992; Plante *et al.*, 1996; Whittington *et al.*, 1999; Miller *et al.*, 2002). Sensitivity of PCR in the present study was relatively low especially for archival paraffin-embedded tissues, while, it has been previously reported 90% (Plante *et al.*, 1996), 71–88% (Whittington *et al.*, 1999) and 76% (Tripathi and Stevenson, 2012) of all samples examined from confirmed positive cases of John's disease by various pathological methods. The sensitivity of PCR on formalin-fixed, paraffin embedded tissue sections has been demonstrated to be dependent on several factors such as type of fixation, the duration of the fixation, length of DNA fragment to be amplified, the age of the paraffin blocks as well as PCR inhibitors in extracted DNA (Greer *et al.*, 1994; Plante *et al.*, 1996; Cox *et al.*, 2006; Groelz *et al.*, 2013). Previous studies have reported that tissues fixed in alcohol and other fixatives such as acetone, used for PCR amplification have shown superior performance over formalin-fixed tissues (Srinivasan *et al.*, 2002; Tripathi & Stevenson, 2012). They showed that many fixatives especially those containing acid, result in a significant decrease in the genomic DNA that

can be amplified. Indeed, acids may hydrolyse glycosidic bands, thus generating abasic sites in DNA, so, tissues fixed in acidic solutions are not considered desirable for amplification. Of the fixatives tested by Greer *et al.* (1994), 95% ethanol, acetone, followed by 10% buffered neutral formalin (BNF) was more successful in PCR amplifications. In addition, the length of duration a tissue sample is kept in a fixative is closely related to the effects of type of fixative used in tissue processing. Previous investigations have indicated that after approximately 24 hours of tissues fixations, the ability to amplify large PCR products decreases with BNF (Greer *et al.*, 1994; Sechi *et al.*, 2001; Srinivasan *et al.*, 2002). They stated that the non-acidic fixatives afford the great amplification of fragments in length and are less damaging to DNA. Indeed, damage on DNA may occur during the fixation process, because variables such as time of fixation or nature of fixative solution can alter the DNA integrity (Greer, 1991; Wolff *et al.*, 2011). The average size of DNA extracted from tissues fixed in buffered formalin decreases with increasing fixation time (Plante *et al.*, 1996; Whittington *et al.*, 1999; Wolff *et al.*, 2011). The most likely factors that influenced the sensitivity of PCR in our study might be the prolonged duration of fixation in 10% formalin degrading Map DNA or inhibitory effects of formalin and particularly paraffin on Taq polymerase activity. Significant inhibitory effects of 10% formalin on PCR amplification of *M. leprae* in biopsy tissues have been previously demonstrated when fixation time exceeded 24 hours (Fiallo *et al.*, 1992). A possible reason for false negative results in PCR can be due to failure of function of the Taq DNA polymerase due to inhibitors such as formalin and paraffin in the

DNA template preparations. Another explanation for such obtained results of the PCR analysis from the paraffin-embedded tissues may be presence of relatively small numbers of acid-fast mycobacterial antigens that were visible in the sections from these tissues particularly in the paucibacillary forms. In addition, it is likely that no *M. paratuberculosis* genome was present in the tissue sections used in the PCR test or that amount of the genome was insufficient to be detected with the agarose gel containing ethidium bromide stain used to detect the PCR product. Additionally, length of the amplified fragment in PCR related to paraffin-embedded tissues affects the result of PCR. Thus, if length of the amplified fragment is big, by increasing the time the samples are kept in formalin, sensitivity of PCR is far less than in cases when the fragment is short (Fiallo *et al.*, 1992). To increase amplification efficiency it is indispensable to select PCR primers that produce products which are as short as possible (Lehmann & Kreipe, 2001). In the cited study, a 300 bp fragment was not detectable, whereas the 80 bp fragment gave a strong band. Therefore this can be a possible reason for the obtained negative results from paraffin-embedded samples in our study could be the fact that the applied fragment (413 bp) was long. Whittington *et al.* (1999) compared the efficacy of amplifying long (413 bp) and short (229 bp) fragments of the IS900 gene in detecting MAP from the paraffin sections and reported higher sensitivity with short fragments (88%) in comparison to long fragments (71%). Also, Greer *et al.* (1994) reported a rapid decline in the success of PCR for fragments greater than 500 bp after 1 to 2 years. In the present study the 413 bp product of IS900 was amplified unsuccessfully after the storage of blocks for

several years. Nevertheless, short (270 bp) fragments of the human betaglobin gene were amplified after storage of paraffin blocks for about 15 years (Sechi *et al.*, 2001). However, it has been suggested that the PCR for detection of *M. paratuberculosis* using the IS900 is a specific and sensitive method for confirmation of Johne's disease (Plante *et al.*, 1996; Whittington *et al.* 1999). In the present study, it seems that using PCR assay on the old paraffin-embedded tissues in comparison with the fresh samples cannot be useful but the low number of samples does not allow making a reliable conclusion.

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