



## Comparative evaluation of FAT, IS900 PCR and microscopy vis-a-vis histopathology for the detection of *Mycobacterium avium* subsp *paratuberculosis* infection in tissues of goats naturally died in herds endemic for Johne's disease

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### ABSTRACT

Study investigated infection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in paired tissues viz. ileum and mesenteric lymph nodes (MLN) from 74 goats using histopathology, acid fast staining of tissue sections, fluorescent antibody test (FAT), IS900 tissue PCR and culture. Histopathology of H&E stained tissue sections revealed variable grades of lesions of Johne's disease (JD) in 37 (50.0%) ileae and 21 (28.4%) MLN. In AFB staining, 16 (21.6%) intestine and 10 (13.5%) MLN revealed presence of acid fast bacilli indistinguishable to MAP. Thirteen (17.5%) intestine and 9 (12.1%) MLN were positive in FAT. Eleven (14.8%) intestine and 7 (9.4%) MLN were positive by IS900 tissue PCR, whereas 7 (9.5%) intestine and 8 (10.8%) MLN were positive in culture. The study showed that sensitivity of histopathology (H&E staining) was highest as compared to other routine tests for the diagnosis of MAP infection in tissues of goats died (irrespective of cause of deaths) during study period from goat herds endemic for Johne's disease. Lower sensitivity of FAT than histology was due to the strong cell mediated immunity exhibited by MAP. Though difference was not significant between sensitivities of FAT and PCR, FAT and AFB staining, and agreement was very good; however, results of the study suggested, that FAT may be a useful test to improve the diagnostic efficacy of caprine Johne's disease and more effective in the detection of grade I lesions as compared to culture and PCR. Study also showed that it was essential to collect both tissues (intestines and MLNs) for the diagnosis of MAP infection at necropsy.

**Key words:** Diagnosis, Goats, Multiple tests, *Mycobacterium avium* subspecies *paratuberculosis*

Johne's disease (JD) is incurable chronic granulomatous enteritis of domestic livestock. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is distributed worldwide and is endemic in native goats (Singh *et al.* 2014, Garg *et al.* 2015). JD slowly progresses to hypoproteinemia, malabsorption, oedema and progressive thickening of gut, which may possibly be the main cause of weight loss (Patterson and Berrett 1969). Diagnosis of MAP is a major challenge. Conventionally histo-pathology, acid-fast staining of tissue (intestines and MLN) smears and sections, and cultivation of bacilli are used for its diagnosis (Chiodini *et al.* 1984). Present study compared the diagnostic efficacy of histopathology with respect to acid-fast staining, fluorescent antibody test, IS900 tissue PCR and tissue culture for the

diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in tissues (intestine and mesenteric lymph nodes) of goats died naturally in goat herds endemic for caprine Johne's disease.

### MATERIALS AND METHODS

*Collection of samples:* Goat herds located at Central Institute for Research on Goats, Makhdoom were endemic for JD, since establishment of institute in 1979. During the period of seven months (January to July 2013), a total of 74 goats were subjected to post-mortem examination after their natural death. In post-mortem house, all these animals were thoroughly examined including small and large intestines. Detailed information about the animals regarding age, sex, significant clinical signs and health status were recorded before necropsy. Age of the goats ranged from 6 to 36 months and majority of the goats examined were females (Table 1). A total of 148 tissue samples (74 ileo-caecal junction and 74 mesenteric lymph nodes) were collected at the time of necropsy from different breeds of goats (Barbari (44), Jamunapari (22) and Jakhra (8)).

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Table 1. Breed-wise and age-wise profile of tissue samples collected from goat herds endemic for Johne's disease

Breed	Age (in months)				Total (n)
	0 - 3	4 - 6	7 - 11	> 12	
Barbari	8	10	10	16	44
Jamunapari	4	5	2	11	22
Jakhrana	1	3	1	3	8
Sub-total	13	18	17	26	74
Cumulative total (n)	13	← 35 →		26	74

These herds were endemic for JD. Half portion of the tissues was collected in 10% buffered formalin for histopathologic examination and another portion in sterilized plastic polybag without adding any preservatives and were kept at  $-70^{\circ}\text{C}$  for microbiological and molecular studies.

**Gross examination and histopathology:** Significant gross lesions including mucosal thickening, mucosal corrugations, prominent lymphatics and intestinal discolorations were examined in lymph nodes and intestines. Tissue samples of intestines and mesenteric lymph nodes were collected and preserved in 10% neutral buffered formalin and subjected to histopathological examination as per previously described procedure (Bancroft and Stevens 1990). Tissue sections (4–5  $\mu\text{m}$ ) were cut and stained with Haematoxylin and Eosin (H&E). Staining of tissue sections by AFS was performed as described previously (Prophet *et al.* 1994). Other part of tissue samples were kept for bacterial culture and molecular assays at  $-70^{\circ}\text{C}$  until processed.

**Grading of lesions:** From 74 ileae and corresponding lymph nodes were observed under 4 $\times$ , 10 $\times$  and 40 $\times$  objectives.

Histo-pathological lesions were recorded, classified and graded as type I, II, III and IV, depending on the type and density of cellular infiltrates (lymphocytes, macrophages and epithelioid cells) in small intestine and mesenteric lymph nodes as per Hailat *et al.* (2010). Lesions having infiltration of lymphocytes in larger amount with very few macrophages and epithelioid cells were considered as grade I. Lesions having infiltration of lymphocytes in lesser amount than the grade I with some macrophages and epithelioid cells (more than the grade I) were considered as grade II. Lesions having small amount of lymphocytes with abundant number of epithelioid cells and macrophages forming micro-granuloma were considered as grade III. Lesions having few lymphocytes and large amount of

epithelioid cells with proliferation of Peyer's patches and formation of micro-granuloma with giant cell were considered as grade IV (Table 2). Tissue sections were considered positive for JD when there were macrophage infiltrations or epithelioid cells obvious in the lamina propria of the villi or sub-mucosa.

**Ziehl-Neelsen acid-fast staining:** Sister sections of formalin fixed paraffin embedded (FFPE) tissues of intestine and mesenteric lymph nodes (MLN) were stained with modified Ziehl-Neelsen's method for demonstration of AFB (Luna 1968). After deparaffinization in xylene, slides were flooded with carbol-fushin, heated gently till steaming and were left for 10 minutes. Slides were then rinsed in water and destained with 3% acid alcohol for 30 sec followed by washing and counter stained with methylene blue for 30 sec. Each ZN acid-fast slide was examined microscopically for the presence of acid-fast bacilli within macrophages or multinucleate giant cells in low (10 $\times$ ) and high (40 $\times$ ) magnification. Slides displaying pink coloured short rods morphologically indistinguishable to MAP under 100 $\times$  oil immersion microscope were considered positive.

**Fluorescent antibody test (FAT):** FAT was carried out in formalin FFPE tissue section as per Gilmour *et al.* (1978). The slides were observed immediately in fluorescent microscope. Epithelioid cells containing MAP giving green fluorescence under fluorescent microscope were considered as positive.

**DNA extraction:** Extraction of DNA from tissue samples was performed as per Sivakumar *et al.* (2005). Mycobacterial DNA was crudely extracted using Tri-reagent (Invitrogen, Germany) from selected tissue (intestine and lymph node) as per manufacturer's instruction.

**IS900 tissue PCR:** PCR was performed using MAP specific IS900 primers (P90 and P91). PCR was performed using specific IS900 primers (P90B 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG -3' and P91B 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC -3') as per Marsh *et al.* (1996)

**Culture of tissue samples on Herrold's egg yolk (HEY) medium:** Culture from tissue samples (intestine and lymph node) was performed using Herrold's egg yolk (HEY) medium as per Singh *et al.* (1996). Slants were examined at weekly up to 120 days and characteristic MAP colonies were recorded as per Whipple *et al.* (1991).

**Statistical analysis:** Agreement in the results obtained for different tests was determined by calculating the kappa statistics. The agreement was considered poor, fair,

Table 2. Grade-wise profile of histopathological lesions in intestines (ileum) and mesenteric lymph nodes of goats

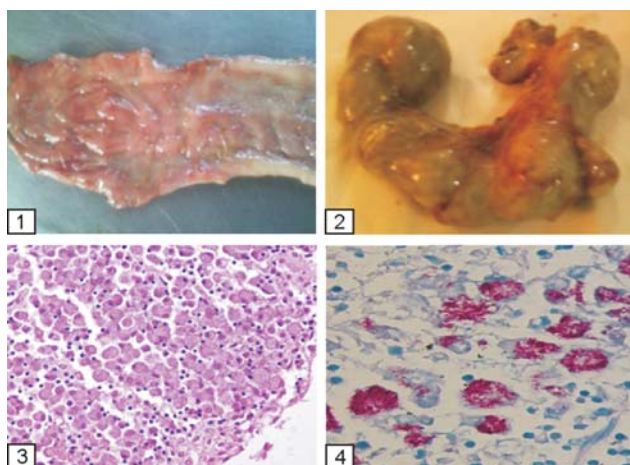
Grade	Lymphocytes	Macrophages	Epithelioid cells	Payer's patches proliferation	Micro-granuloma
I	++++	+	+	-	-
II	+++	++	++	-	-
III	++	++	+++	Yes (moderate)	Yes
IV	+	+++	++++	Yes (severe)	Yes, with giant cell

moderate, good (substantial) and very good (almost perfect) if estimated Kappa values were 0.0-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80, and 0.81-100, respectively. The sensitivity of a test was calculated as per the formula given below.

$$\text{Sensitivity (\%)} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

RESULTS AND DISCUSSION

Histopathological examination of routine stained tissue sections from the 74 ileae and corresponding lymph nodes revealed variable grade of lesions of JD in 37 (50.0%) ileae and 21 (28.3%) mesenteric lymph nodes (Table 3). The most significant gross findings were serous atrophy of the fat and mild to moderate, diffuse thickening of terminal ileum especially at ileo-caecal junction with or without transverse/longitudinal corrugations. In most of the cases, ileo-caecal valves were consistently thickened (Fig. 1). Enlarged mesenteric lymph node showed lympho-follicular hyperplasia causing thickening of cortical area of lymph nodes (Fig. 2). Twenty two (29.7%) intestine and 14 (18.9%) MLNs showed lesions of grade I characterized by infiltrations of lymphocytes in larger amount with few macrophages and epithelioid cells without fusion and formation of micro-granuloma. Nine (12.1%) intestine and 4 (5.4%) MLNs showed lesions of grade II featured by infiltration of lymphocytes in lesser amount with abundant number of macrophages and epithelioid cells without forming distinct granuloma (Table 3). Five (6.7%) and 2 (2.7%) cases of intestine and mesenteric lymph nodes, respectively showed the lesions of grade III characterized by severe infiltration of epithelioid cells with few macrophages forming microgranuloma with very less number of lymphocytes along with moderate proliferation



Figs 1-4. 1. Small intestine showing velvety thickening with folded mucosa. 2. Mesenteric lymph node showing marked enlargement. 3. Ileum showing infiltration of few lymphocytes with large number of epithelioid/mononuclear cells forming sheet like appearance microgranuloma (H&E, 400x) 4. Intestine showing pink colour acid fast bacilli individually or in clusters in epithelioid cells. (Ziehl Neelsen staining, 100x).

of Peyer’s patches (Table 2). Whereas lesions of grade IV in each case from intestine (1.3%) and mesenteric lymph nodes (1.3%), showed infiltration of lymphocytes, macrophages and abundant epithelioid cells forming sheet like appearance in lamina propria and sub-mucosa of the intestine along with severe proliferation of peyer’s patches. Multinucleated giant cells near granuloma were also seen in mesenteric lymph nodes (Table 3; Fig 3). Two cases (each from intestine and lymph node) were considered as IV grade (special category) where calcification, typical granulomatous structure were observed in the lymph nodes. The cases in grades I and II were considered suspected whereas the cases in grades III and IV were considered positive for the active disease by histopathological evaluation.

The tissue samples from the intestine (74 samples) and mesenteric lymph nodes (74 samples) were sectioned and stained by acid fast staining only 16 (21.6%) samples from intestine and 10 (13.5%) samples from mesenteric lymph nodes revealed presence of acid fast pink colour bacilli individually or in clumps in the epithelioid cells



Fig. 5. *Mycobacterium avium* subsp. *paratuberculosis* specific amplicons (413 bp) by PCR using IS900 specific primers. Lane M, 100 bp DNA ladder; Lane 1, positive control; lane 2-19: test DNA samples.

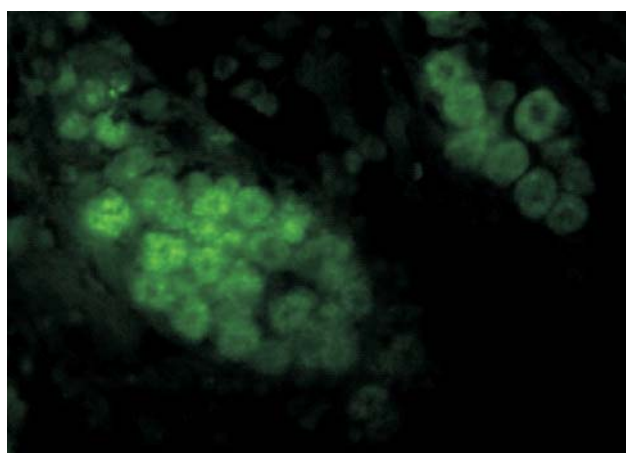


Fig. 6. Clusters of MAP infected epithelioid cells in lamina propria of villi in terminal ileum showing green fluorescence. (FAT 400x).

Table 3. Grades of histopathological lesions and profile of distribution of intestines and mesenteric lymph nodes

Tissue type	n	Grade of lesions [n (%)]				Cumulative total {n (%)}	
		I	II	III	IV	Positives	Negatives
Intestines (ileum)	74	22 (29.7)	9 (12.1)	5 (6.7)	1 (1.3)	37 (50.0)	37 (50.0)
Mesenteric lymph nodes	74	14 (18.9)	4 (5.4)	2 (2.7)	1 (1.3)	21 (28.3)	53 (71.6)
Total Tissues	148	36 (24.3)	13 (8.7)	7 (4.7)	2 (2.7)	58 (36.7)	90 (60.8)

Table 4. Correlation between microscopy (ZN staining), FAT and IS900 PCR in different grades of pathological lesions for the detection of MAP in tissues of goats during the course of disease

Grade of lesion	Tissues (intestines + MLN) positive by histopathology [n (%)]		Tissues (intestines + MLN) positive by FAT, PCR and culture in respective grade [n (%)]		
	H&E staining*	ZN staining	FAT	PCR	Culture
I	36	6 (16.7)	4 (11.1)	1 (2.7)	0 (0.0)
II	13	8 (61.5)	8 (61.5)	8 (61.5)	6 (46.1)
III	7	7 (100.0)	7 (100.0)	7 (100.0)	7 (100.0)
IV	2	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)

\*n = 58/148. In three goats, no histopathological lesions were found.

indistinguishable to MAP (Fig. 4, Table 5). While, 6/36 (13.8%), 8/13 (61.5%), 7/7 (100%) and 2/2 (100%) cases were found AFB positive from grade I, II, III and IV respectively (Table 4). The bacilli were seen either inside the macrophages or outside of the cells.

Of 74 intestine and 74 mesenteric lymph nodes samples cultured only 7 (9.5%) and 8 (10.8%) were positive by culture test which is considered as the gold standard method for detection of *Mycobacterium* (Table 5; Fig. 4). MAP colonies were small to large, flat pinpoint and off white in colour which developed in 3–4 months on HEY slants. In order to further confirm the presence of MAP, intestine and mesenteric lymph nodes tissues samples were subjected to IS900 PCR, and 11 (14.8%) and 7 (9.4%) paired tissue samples of intestine and mesenteric lymph nodes were found positive, respectively (Table 5; Fig. 6). In grade III and IV, PCR results recorded 100% positive, and in grade II, 61.5% tissue samples were positive; whereas 2.7% samples were positive from grade I tissue samples (Table 4). To understand the cellular reaction in the intestine (lamina propria of villi in terminal ileum) and in the respective mesenteric lymph nodes and to observe whether this reaction was associated with the presence of MAP, fluorescent antibody test was used. A positive reaction was

able to be seen by the presence of green fluorescence around the infected epitheloid cells in lamina propria of villi in terminal ileum and mesenteric lymph nodes. In this study, only 74 ilea tissue sections and 74 tissue sections from the corresponding lymph nodes were examined; and 13 (17.5%) from intestine and 9 (12.1%) from MLN were found positive, as green fluorescence was observed (Table 5; Fig. 6). While, 6 (16.7%), 8 (61.5%), 7 (100%) and 2 (100%) tissues were found positive by FAT from grade I, II, III and IV, respectively (Table 4).

Of 36 grade-I cases, 6 (16.7%), 4 (11.1%) and 1 (2.7%) cases were diagnosed positive by ZN staining, FAT and PCR, respectively, and none by culture. Of 13 grade II cases, 8 (61.5%) cases were positive by ZN staining, FAT and PCR and 6 (46.1%) by culture. All grade III and grade IV cases (7 and 2, respectively) were positive in ZN staining, FAT, PCR and culture.

The agreement between histopathology (H&E staining) and ZN staining was moderate. Sensitivity of H&E staining was 88.4% as compared to ZN staining (39.6%). The agreement between (H&E staining) and FAT was observed to be fair. Sensitivity of H&E staining was 95.4% in comparison to FAT (36.2%). The agreement between H&E staining and PCR was fair. Sensitivity of H&E staining was

Table 5. Comparative analysis of different diagnostic tests for diagnosis of MAP infection in tissues of goats

Tissue	Type of tests				
	Histopathology		FAT positive n (%)	IS900 PCR positive n (%)	Culture positive n (%)
H&E staining positive n (%)	ZN staining positive n (%)				
Intestine(74)	37 (50.0%)	16 (21.6%)	13 (17.5%)	11 (14.8%)	7 (9.4%)
MLN(74)	21 (28.4%)	10 (13.5%)	9 (12.1%)	7 (9.4%)	8 (10.8%)
Total(148)	58 (39.1%)	26 (17.5%)	22 (14.8%)	18 (12.1%)	15 (10.1%)

100% in comparison to PCR (31.0%). The agreement between the ZN staining and PCR was good. Sensitivity of ZN staining was 100% in comparison to PCR (69.2%). The agreement between ZN staining and FAT was very good. Sensitivity of FAT was 90.9% in comparison to ZN staining (76.9%). The agreement between FAT and PCR was also very good. Sensitivity of FAT was 100% in comparison to PCR (81.8%).

Moreover, when FAT and acid fast staining were compared, FAT identified 11 of 12 of the same goat and one animal additionally (Table 5). A significant difference was observed in the sensitivity of FAT compared with ZN acid fast staining and PCR for the identification of infected animals. These tests were found to be in perfect agreement, with a kappa value of 0.80 (95% confidence interval = 0.669 - 0.934) and 0.88 (95% confidence interval = 0.774 to 0.995) for ZN and PCR respectively, against FAT (Table 6).

In absence of frank clinical symptoms, diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* is a challenge due to prolong incubation, complex biology and pathogenesis, intracellular nature of bacilli, and poor sensitivity and specificity of diagnostic tests for the detection of sub-clinical infection. Identification of sub-clinically infected animals is difficult as there is variation in the shedding of MAP with the stage of infection (Chiodini *et al.* 1984). Isolation of MAP is easy to carry out from foetus and demonstration of antibodies has been done in animals deprived of colostrums (Jones *et al.* 1997, Lambeth *et al.* 2004).

In the present study, gross lesions, pathological lesions and the occurrence of subclinical Johne's disease was studied in naturally endemic animals using several diagnostic tests, such as histopathological and fluorescent antibody test, AFS, PCR and culture. However, substantial debate exists over which diagnostic test is most effective and sensitive for JD. In the present study, 14.8% (11/74) animals (age >12 months) showed gross lesions indicating Johne's disease (JD) in intestine and mesenteric lymph nodes (MLN). Gross lesions were confined to terminal ileum and mesenteric lymph nodes (MLN). Gross lesions could not be seen in the duodenum, colon, rectum and other

organs in accordance with the previous findings (Kurade *et al.* 2004) in experimental ovine paratuberculosis. The most significant gross findings were serous atrophy of the fat and mild to moderate, focal/ diffuse thickening of terminal ileum especially at ileo-caecal junction with or without transverse/longitudinal corrugations. The mesenteric lymph nodes were severely enlarged approximately double in the size and were found to be oedematous and juicy (Figs 1, 2).

In present study, 50.0% (37/74) intestine and 28.4% (21/74) MLN showed the various grades of histopathological lesions which were characterized on the basis of infiltration of mononuclear cells consisting of lymphocytes, macrophages and epithelioid cells in lamina propria and submucosa of intestine and cortex and medulla of mesenteric lymph nodes with or without formation of microgranuloma. Affected part of the intestine revealed degeneration and partial to complete denudation of lining epithelial cells forming naked villi in this study, as reported previously (Paliwal and Rajya 1982, Rajkumar 1998).

In a previous study, the villi exhibited variable changes that included dilated lacteals, villous distortion and thickening and fusion of villi were observed. Munjal *et al.* (2005), observed similar lesions such as thickening of the intestinal villi with flat and wide tips, infiltration of lymphocytes, macrophages and epithelioid cells in lamina propria of intestine were observed in this study. Mesenteric lymph node showed lymphofollicular hyperplasia causing thickening of cortical area of lymph nodes with variable degree of infiltration of lymphocytes, macrophages and epithelioid cells with or without formation of microgranuloma (Fig. 3).

In this study, grading criteria was adopted similar to Hailat *et al.* (2010) with some modifications. Grade I lesions were seen in 29.7% (22/74) intestines and 18.9% (14/74) MLNs (Table 3) characterized by the infiltration of large number of lymphocytes with few epithelioid cells in mucosa / sub-mucosa of intestines. It has been suggested that these lesions represent early stage of infection and persistence of MAP results in a continuous antigenic stimulation and recruitment of inflammatory cells (Allen *et al.* 1974). The grade II lesions were found in 9 (12.1%) intestine and 4 (5.4%) mesenteric lymph nodes and characterized by infiltration of large number of macrophages/epithelioid cells with fewer lymphocytes. Lymphocytic and macrophage infiltrations were inversely correlated with each other, suggesting the occurrence of antagonistic pathological processes and macrophage infiltration was much correlated with the presence of acid-fast bacteria (Clark *et al.* 1996). Moreover, in this study, 5 (6.7%) cases from intestine and 2 cases (2.7%) from mesenteric lymph nodes showed the grade III histologic lesions, while, only 1 (1.3%) animal showed grade IV histopathologic lesions. Grade III and IV histologic lesions characterized by severe infiltration of epithelioid cells with formation of distinct micro-granuloma and Giant cells were also seen in grade IV lesions (Table 3). Grade II and IV lesions noted in this study were similar

Table 6. Statistical evaluation of tests for the detection of MAP infection in tissue sections

Test	Kappa value	95% confidence limit
Histopathology (H&E staining) vs. ZN staining	0.403	0.263 - 0.542
Histopathology (H&E staining) vs. FAT	0.394	0.259 - 0.530
Histopathology (H&E staining) vs. PCR	0.354	0.223 - 0.484
ZN staining vs. PCR	0.788	0.648 - 0.928
ZN staining vs. FAT	0.801	0.669 - 0.934
FAT vs. PCR	0.885	0.774 - 0.995

to pigmented multi-bacillary group as described previously in multibacillary cases of ovine paratuberculosis (Clark *et al.* 1996), type IIIa and IIIb of Perez *et al.* (1996) and grade II and IV lesions of Kurade *et al.* (2004). Gross lesions were clearly visible in lesions of grade III and IV. However, another study reported that gross lesions were clearly visible only in sheep with type 3b and 3c lesions (Perez *et al.* 1996). Grade I lesions noted in our study corresponded to some extent with pauci-bacillary form of Clark *et al.* (1996), group II lesions of Stamp and Watt (1954), and Rajya and Singh (1961); grade I lesions of Hailat *et al.* (2010), and Kurade *et al.* (2004); and type I and III lesions of Perez *et al.* (1996).

Accumulation of macrophages and epithelioid cells and formation of granuloma in cortex and medulla suggested that the mycobacteria might have entered in the lymph nodes through afferent lymphatic subsequently the infection extended to paracortex through subcapsular sinuses and then migrated to medullary areas in later stage of infection. The distinct granuloma at places, were formed and follicles became depleted after chronic outflow of lymphocytes in the lymph node conform the findings of several workers who reported similar development of lesions in lymphoid organs in paratuberculosis in sheep and goats (Paliwal and Rajya 1982, Perez *et al.* 1996). In present study, mucosal thickening and corrugations occurring was suggested due to mononuclear cell infiltration. Similarly, inflammatory cell infiltrates were responsible for much of the increased thickness in the mucosa and submucosa and for the villous atrophy (Clark *et al.* 1996, Al-Dubaib and Mahmoud 2008).

In present study, only 26 (17.5%) of histopathological tissue samples out of 148 cases revealed the presence of acid-fast bacilli, including 16 (21.6%) from intestine and 10 (13.50%) from MLN (Table 5). In accordance with our findings, Clarke *et al.* (1996) reported 50% paucibacillary cases positive in AFB staining. However, we observed that 5/36 (13.8%), 8/13 (61.5%), 7/7 (100%) and 2/2 (100%) cases were found AFB positive from grade I, II, III and IV, respectively (Fig4). Though in grade I lesions, positivity in ZN staining was very low, detecting only 6 (16.7%) sections as positive. These results were in accordance with the findings of Kurade *et al.* (2004) who reported fewer or no AFB in grade I lesions of sheep. AFB were detected histologically in 61.5% of grade II lesions. Similar observations were reported by Stamp and Watt (1954). Similarly, Clarke *et al.* (1996) reported 50.0% pauci-bacillary cases positive in AFB staining. It may be explained on the basis of strong cell mediated immunity (CMI) reported in ovine and caprine JD (Clarke *et al.* 1996).

*Mycobacterium avium* subspecies *paratuberculosis* was cultured from intestine and MLN tissues of all the animals with grade III and IV lesions and 6/13 (46.15%) were also culture positive from those with grade II lesions, and no culture was reported from grade I lesions in present study (Table 4). This may be due to reduced bacterial viability caused by freezing and thawing of the specimens. These results were in accordance with the previous study reported

by Kurade *et al.* (2004), who observed detectable growth of MAP in tissue samples from the infected sheep. Amongst the diagnostic tests for paratuberculosis, it was noted that sensitivities were lower of bacterial culture of infected tissues as compared to histologic demonstration of bacilli. However, diagnosis of paratuberculosis by culture is mostly hampered by long incubation period, relatively low sensitivity and contamination (Merkal *et al.* 1987). It may also be attributed to the existing limitations of conventional culture method, especially in small ruminants (Carrigan *et al.* 1990, Sigurdardottir *et al.* 1999, Whittington and Sergeant 2001). In accordance with our findings, low sensitivity of the culture had been reported by many workers (Chiodini *et al.* 1984, Van der Giessen *et al.* 1992, Collins *et al.* 1996, Whitlock *et al.* 2000, Miller *et al.* 2002) and it may be due to the presence of small number of bacilli or presence of spheroplastic form of bacteria in the tissues. It is possible that harsh decontamination step in the bacterial culture may also be responsible for the poor sensitivity (Sivakumar *et al.* 2005). Similarly, activity of growth promoter (mycobactin J), also has major impact on outcome of culture (personal experience of 27 years of author involved in primary isolation of MAP).

Direct detection of MAP can be done from quarter milk and bulk tank milk by IS900 PCR (Pillai and Jayarao 2002). In this study also, tissues were subjected to IS900 PCR to detect MAP specific DNA. All (1000%) the grade III and IV tissues were positive in IS900 PCR, and 2.7 and 61.5% were positive in grade I and II, respectively. It was possibly because of insufficient test sensitivity or a genuine absence of MAP in the samples. Variation in amplification from grade I and II lesions could be due to the presence of large amount of host cell DNA that reduce the actual concentration of bacterial DNA and thereby insufficient template DNA in PCR mixture (Gwozdz *et al.* 1997). To avoid this, the bacterial DNA should be concentrated. It will enhance the sensitivity by increasing the availability of more bacteria from large quantity of tissue and by decreasing the unwanted host cell DNA. Therefore, purification step may be incorporated in PCR test when handling sub-clinical / pauci-bacillary cases containing limited number of organisms (Sivakumar *et al.* 2005). PCR amplification using IS900 primers against MAP DNA was most reliable method for the diagnosis of infected animals.

In our study, it has been observed that FAT technique is more reliable than IS900 PCR to detect MAP from the grade I animal tissue samples. IS900 PCR has been used to detect small amounts of MAP DNA in faeces (Fang *et al.* 2002) and tissue including culture negative tissues samples (Miller *et al.* 2002). PCR analysis previously performed by our laboratory demonstrated that IS900 was specific for MAP and found reliable detection of MAP. But in grade I tissue samples only 2.7% positive samples may be indicative of low amount or absence of Mycobacteria inside the tissue (Ellingson *et al.* 2000, Miller *et al.* 2002).

Diagnosis of the grade I cases was found to be difficult using FAT and PCR tests and there was little likelihood of

detecting AFB in such tissues. Low mycobacterial load in grade I cases seemed to be associated with poor humoral immunity. In mycobacterial diseases, various antibody-helper, delayed-type hypersensitivity, cytotoxic or even suppressor roles have been suggested for infiltrating lymphocytes (Modlin *et al.* 1986, Arnoldi *et al.* 1990). Presence of intestinal lesions with low mycobacteria count suggest an immuno-pathological mechanism may be involved in MAP infection (Clarke *et al.* 1996). Hermon-Taylor (1998) reported that the pauci-bacillary form of the disease occur particularly in sheep and goats and in such animals, MAP cannot be detected in the tissue microscopically and usually cannot be cultured.

Immunohistochemical techniques are potentially more sensitive and specific than ZN acid-fast staining due to ability to distinguish not only intact viable organisms but also soluble MAP, cell fragments, and altered microorganisms with defective cell walls (Plante *et al.* 1996). Immunoperoxidase staining was found better than acid-fast staining as it revealed small numbers of bacteria in bovine tissue sections (Brees *et al.* 2000). Thirteen (17.5%) cases from intestine and 10 (12.1%) cases from MLN were found positive by FAT (Table 5) as green fluorescence (Fig. 6). No significant difference was found in the sensitivities of ZN acid-fast staining and FAT, with only one additional positive result obtained when using FAT compared to ZN acid-fast staining (Table 5). After kappa analysis, these tests illustrate considerable agreement, identifying the most of the similar infected animals.

In our study, all the tissue sample were found positive by IS900 PCR analysis for specific MAP DNA in grade III and grade IV, and a majority of grade I and II were negative, possibly because of insufficient test sensitivity or a genuine absence of mycobacterial in the sample (Ellingson *et al.* 2000). However, Histologic AFB staining detected more infected animals as positive as compared to bacterial culture, FAT and PCR. The PCR test was found to be better than bacterial culture in grade I. Diagnosis of the grade I cases was more difficult because the fluoroscent antibody test and polymerase chain reaction tests were usually negative and there was little likelihood of detecting acid-fast organisms in tissue. The very low mycobacterial load of grade I cases would seem to be associated with poor humoral immunity. However, the substantial lymphocyte recruitment into the gut of grade I cases would seem consistent with a strong cell-mediated immune response. We did not investigate whether the lymphocytic infiltration was caused by the low acid-fast presence, nor did we investigate the function of the lymphocytes.

In this study, no significant difference was found between the sensitivity of FAT and PCR and between the sensitivity of FAT and ZN staining as agreement was very good. However, sensitivity of FAT was less than the histological H&E staining for the diagnosis of MAP infection in goats. In contrary to our findings, Stabel *et al.* (1996) reported high sensitivity of immuno-histochemical staining for the detection of JD in the infected bovine tissues. Since we

used a polyclonal antibody, it is unfair to consider this antibody completely specific for MAP, so this may be the reason of low sensitivity of FAT. Furthermore, other researchers have also reported that polyclonal and monoclonal antibodies could be used for the diagnosis of JD in tissue sections (Coetsier *et al.* 2000). Lowered sensitivity of FAT than histological H&E staining is also related to the strong CMI which clear mycobacterial load in early sub-clinical infection.

In this study, sensitivity of histopathology (H&E staining) was higher as compared to other tests for the diagnosis of MAP infection in goats. Similar to our findings, Perez *et al.* (1996) and Clarke *et al.* (1997) reported that histological lesions in JD are characteristic, and, if present, confirm the diagnosis. Intestines were better tissue as compared to MLN; however, it is better if both the tissues are sampled and treated as individual samples (Vohra *et al.* 2008).

There was no significant difference between sensitivity of FAT and PCR and sensitivity of FAT and acid fast ZN staining (agreement was considered to be very good). However, sensitivity of FAT was lesser than histological H&E staining for the diagnosis of MAP infection in goats. In contrary to our finding, Stabel *et al.* (1996) reported high sensitivity of immunohistochemical staining for the detection of Johne's disease in infected bovine tissue. We used a polyclonal antibody but indicating low sensitivity of FAT due to the low number of samples. Furthermore, others have also reported that polyclonal and monoclonal antibodies could be used for the diagnosis of paratuberculosis in tissue sections (Coetsier *et al.* 1998). Lower sensitivity of FAT than histological H&E staining is also related to the strong CMI which clear mycobacterial load in early subclinical infection. The study suggested, that FAT may be a useful technique to improve diagnostic efficacy of caprine Johne's disease and more effective in detection of grade I samples as compared to culture and PCR. In addition, these cases were tested positive by FAT using a specific rabbit polyclonal antibody to *M. paratuberculosis* antigens. Thus, we consider Johne's disease to be a major crisis in goats in endemic area and believe there is a greater need for additional studies on prevalence and epidemiology in order to control JD in goat population.

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