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Pathobiology of Spontaneous and Experimental Paratuberculosis (S-5 strain) in Goats with Special Reference to Early Lesions

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ABSTRACT

The present study was aimed to diagnose early cases of paratuberculosis in goats by demonstration of Acid Fast Bacteria (AFB) in faecal and tissue samples; isolation of organisms from faecal and tissue samples, enzyme linked immunosorbent assay (ELISA) and patho-morphological lesions in experimental infection using "Indian Bison Type" biotype strain S-5 of *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Faecal samples from 142 goats from various farm herds of North India were subjected to smear (using centrifugation and decontamination) and cultural examinations. Isolation of MAP was performed in all faecal and 74 tissue samples by inoculation on Herrold's Egg Yolk (HEY) medium with or without Mycobactin-J after decontamination with 0.9% Hexadecylpyridinium Chloride (HPC). Experimental study was conducted on 13 young goats (10 infected, 3 controls) where pathogenicity of the strain S-5 was tested by gross and histopathological lesions and plate-ELISA test. Characteristic gross and microscopic lesions were observed at 90 Days Post Infection (DPI) and onwards. Lesions showing infiltration of macrophages with AFB without granuloma formation, simulating lepromatous form of human leprosy and typical granuloma as in tuberculoid form were observed. Positive humoral immune response was observed at 90 DPI onwards showing antibody titer above the cut off value. There was apparent linear correlation between the antibody levels and days post infection. Performance of different diagnostic tests like examination of faecal smear by direct microscopy, faecal culture, scraping smear examination for MAP from tissue, pathomorphology and plate ELISA test had linear relationship among them. Such study ultimately may help the researchers to select the specific series of tests for detection of MAP from clinical samples.

Key words: *Mycobacterium avium* ssp. *paratuberculosis,* paratuberculosis, goats, pathobiology, agar gel immunodiffusion, ELISA, Johnin test

INTRODUCTION

Mycobacteria are either saprophytic or obligatory ancient microbes (Ventura *et al*., 2007; Hett and Rubin, 2008; Deb *et al*., 2012). Paratuberculosis, commonly known as Johne's Disease (JD), is typically a chronic granulomatous disease caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP). It is primarily an intestinal ailment which is deadly in nature and leads to chronic enteritis as well as granulomatous inflammation of the lymph nodes in ruminants (Pant *et al*., 2011; Deb and Goswami, 2011). The study was focussed on the development of disease in small ruminants particularly goats; which are the usual host for being a source of livelihood to large section of resource less or resource poor majority in India. Goat is primarily an Asian animal and JD has not been so well studied in goats as in the case of cattle and sheep. Therefore, most of the studies conducted on cattle have been assumed to hold true for goats also. Prevalence of caprine paratuberculosis in India appears to vary greatly in different herds in different localities and ranges from 2-22% (Singh *et al*., 1996; Tripathi, 2008). Productivity as well as viability of animals industry is adversely affected by the disease across the world, thereby causing significantly high economic losses by of reduced productivity; higher culling rate at premature age as well as increased mortality (Pant *et al*., 2010, 2011). In India, most of the studies to estimate prevalence of MAP have been based on Johnin test, which has low reliability and has been largely questioned (Paliwal, 1984). Therefore, an attempt was made to study the prevalence of spontaneous cases of caprine paratuberculosis in farm herds using pathological, bacteriological and serological methods. Diagnosis of paratuberculosis is difficult during early and subclinical stages of the infection. Disease in the clinical stage is generally diagnosed by detection of organism in fecal smear; bacterial culture and Polymerase Chain Reaction (PCR); detection of antibody response to MAP infection by enzyme linked immunosorbent assay (ELISA), agar gel immunodiffusion test (AGID) and the detection of cell mediated immune response (CMI) by Johnin test and gamma interferon assay (Collins, 1996; OIE, 2000; Singh *et al*., 2014). Therefore, the present study was designed to diagnose early cases of Paratuberculosis in goats by demonstration of Acid Fast Bacteria (AFB) in faecal and tissue samples; isolation of organisms from faecal and tissue samples; patho-morphological lesions/pathobiology in experimental infection using 'Indian Bison Type' biotype strain S-5 of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and knowing relationship of the development of pathomorphological lesions with the immune response using Plate-ELISA.

MATERIALS AND METHODS

Spontaneous cases of JD were studied in a total of 51 goats, aged 10 day to 6 years, from 10 different farm herds and submitted to the Division of Goat Health, Central Institute for Research on Goats (CIRG) for necropsy diagnosis. A total of 142 faecal samples of goats of various age groups and of either sex from different farm goat herds were subjected to direct microscopic and cultural examination. Fecal samples (142) from ileocaecal valve region and mesenteric lymph nodes were collected from dead animals suspected for paratuberculosis. Processed tissue samples were decontaminated with 0.9% Hexadecile Pyredinium Chloride (HPC), concentrated and inoculated on Herrold's Egg Yolk Medium (HEYM) with and without Mycobactin-J.

In the experimental part of the study, thirteen Barbari kids of 4-6 weeks of age of either sex were divided into two groups (10 kids in infected group-I and rest 3 in control group-II). The kids of group-I were infected with well characterized 'Indian Bison type' strain 'S-5' of *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The strain 'S 5' was characterized on the basis of specific MAP probes. Actively growing week old culture of S-5 strain at VIII-IX passage level was

harvested from six Mc Cartney bottles by gently scrubbing with swab in sterilized normal saline. The live MAP culture (125 mg wet weight) suspended in 1 L of pasteurized goat milk was fed orally at 200 mL/kid with bacterial concentration 10^7 mL^{-1}. Experimentally infected kids were studied at the intervals of 30, 60, 90 and 120 Days Post Infection (DPI) for isolation, cultural examination, demonstration of Acid Fast Bacilli (AFB), serological test and pathomorphological studies.

The culture of MAP was subpassaged on solid HEY medium with Mycobactin-J (Allied Monitor Inc., Fayette, USA). During the course of study, clinical symptoms were examined daily and direct microscopy and cultural examination of faeces and tissue samples were performed. Humoral immune response was assessed by plate ELISA test and gross and histopathological studies done using staining with H and E as routine and Ziehl Neelsen (ZN) as special stain at 30 days interval. For detection of humoral immune response, US protoplasmic antigen (Ag) (Allied Monitor Inc. Fayette, USA) (commercial antigen) was prepared by diluting 10 unit vial of the purified protein antigen in 1 mL of sterilized Triple Distilled Water (TDW) and dispensed in aliquots of 0.75 mL in sterilized eppendorf tubes stored at -200°C. Plate ELISA test was carried out as per the method described by Milner *et al.* (1987) using commercial protoplasmic antigen. Optimum concentration or dilution of antigen (Ag) and antibody (Ab) conjugate were determined by checker board analysis. Anti-goat rabbit IgG-peroxidase conjugate (Bangalore Genei, India) as anti antibody and ortho phenyl diamine dihydrochloride (OPD) as substrate were used. Absorbence was read at 450 nm in ELISA reader (Multi scan, Thermolab System, Finland). Serum samples having absorbance equal to more than the mean average plus twice the Standard Deviation (SD) of the negative serum samples were recorded as positive. The cut off value calculated for unabsorbed ELISA test was found to be 0.235 using commercial antigen of US origin.

RESULTS

Bacteriology

Spontaneous cases: Of the 142 faecal smears examined, 52 (36.6%) were positive for presence of typical AFB and were considered positive for MAP infection. In culture, 26 (18.3%) samples exhibited typical colonies suggestive of MAP on Herrolds Egg Yolk Medium (HEYM) with Mycobactin J. Besides this, 4 (2.8%) faecal samples showed the growth of atypical Mycobacteria (fast growers) on HEY media. In ZN staining of smear prepared from ileo-caecal junction and Mesenteric Lymph Nodes (MLN), 23 (31.08%) out of 74 samples were positive showing typical pink stain acid fast organism (Fig. 1).

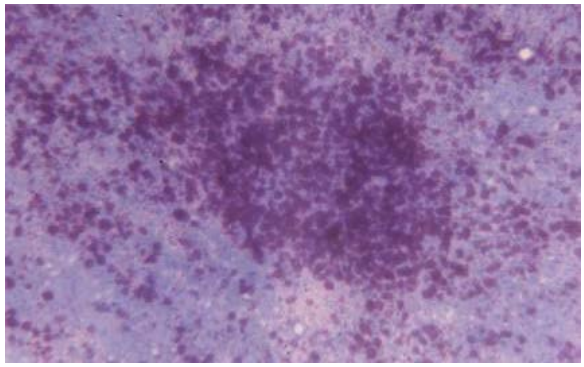

Fig. 1: Scraping smear from ileo-caecal junction showing typical AFB. Ziehl-Neelsen stain 100X

In a few cases, congregation of AFB and or clumps of degenerated AFB were also observed. Of the 51 animals, 37 Mesenteric Lymph Nodes (MLN) and 37 ileo-caecal tissues (ICJ) processed and inoculated on HEY mediums slants with and without Mycobactin J revealed isolation of typical colonies in 7-8 weeks in 14 (37.84%) MLN and 14 (37.84%) ICJ. Colonies on HEY medium were semi-spherical with entire margin and straw coloured, varying from 3-4 mm in size. Colonies had nipple in the center around the flat area. Besides typical colonies, 4 isolates were of atypical Mycobacteria (fast growing) which grew within 3 weeks time.

Experimental cases: Two kids in the infected group I and one kid in the control group were positive in faecal smear examination and faecal culture, respectively on 60 days post infection (DPI). Three kids were positive in both faecal smear and faecal culture after 3 months post infection. In the positive cases, one was multi bacillary and had more than 10 colonies and two were pauci bacillary showing less than 10 colonies of MAP. All the 3 infected kids were found positive in faecal smear and faecal culture examination after 4 months of post infection. Almost all the infected group of experimental kids periodically showed the presence of typical AFB in direct microscopy of scraping smear examination. Typical colonies suggestive of MAP were observed in one kid on 30 DPI, 2 cases on 60 DPI, in 2 cases from tissue of ICJ and 3 cases from MLN on 90 DPI and all the 3 animals on 120 DPI.

Serology for detection of humoral immune response: The plate ELISA testing, employing commercial US antigen, revealed that no kid was found serologically positive on 30 and 60 DPI. Positive sero- reactivity was observed at 90 and 120 DPI at 450 nm with OD value ranging between 0.23-0.37 (minimum) and 0.82-1.25 (maximum) in ELISA test; when sera was assessed against US antigen having the estimated cut off value 0.235.

Pathomorphology: Two kids, each sacrificed on 30 and 60 DPI, revealed no characteristic gross lesions in the different segments of small intestine. Mesenteric lymph nodes were slightly enlarged and oedematous. Firmness of liver was observed in one case at 60 DPI. Other organs revealed no significant changes. Microscopically, no characteristic lesions were observed in intestines at 30 and 60 DPI. In the Mesenteric Lymph Nodes (MLN), only oedematous fluid was found to be present in medullary region in ZN stain tissue sections and no AFB were demonstrated in the intestines and lymph nodes of any sacrificed animals at 30 and 60 DPI. Three infected and one control groups of kids sacrificed on 90th DPI grossly revealed emaciation, increased straw colour peritoneal fluid and pale mucous membrane. Mild thickening and characteristic transverse rugous folding simulating the convolution of the cerebrum of the intestinal mucosa was observed. Transverse corrugation was prominent from the serosal surface of the ileum and jejunum (Fig. 2 and 3).

Corrugation of the small intestine was distinguishly prominent in the posterior part of the ileum. The MLN were enlarged and oedematous and the size was increased from 2 cm up to 15 cm. Other abdominal lymph nodes namely jejunal and illeo-caecal were also enlarged and oedematous. Cut surfaces were paled with poor cortico medullary distinction in few cases. Lymphatic along the MLN were enlarged and oedematous. Slight firmness was observed in liver; however spleen, lungs, kidneys, uterus, testes, ovary, mammary gland, aorta, venacava and endocardium did not reveal any significant lesions. Microscopically, intestinal villi showed mild to moderate disruption and desquamation of epithelial lining. Infiltration of a large number of lymphocytes, plasma cells and macrophages were seen in the areolar tissue of lamina propria. Lymphocytes were prominently

Fig. 2: Generalized thickening and transverse foldings of the intestinal mucosa on 90 DPI

Fig. 3: Generalized thickening and transverse foldings of the intestinal mucosa on 105 DPI

present and occasionally the area also revealed infiltration of epitheloid cells and macrophages forming loose aggregates or foci. Submucosa revealed moderate infiltration of mononuclear cells. It was oedematous and tunica muscularis was slightly disrupted. Infiltration of lymphocytes and large number of macrophages and/or epitheloid cells were also observed. Serosa was mildly thickened and infiltrated by a few mononuclear cells with dilated lymphatics. Cortical and paracortical region of MLN showed infiltration mononuclear cells, macrophages having vesicular nucleous and containing hemosiderin pigments. Loose aggregates of macrophages along with oedematous fluid were noticed in medulla. Grossly, all the 3 animals of infected group, sacrificed on 120 DPI, showed thickening of both longitudinal and transverse folding of corrugations together in jejunum and ileum; which were prominently observed from serosal surface. The MLN and illeo caecal lymph nodes were enlarged, oedematous and knotted and corded with soft consistency and poor cortico medullary distinction. Microscopically, the jejunum and ileum villi were short, broad and club shaped with infiltration of lymphocyte, plasma cell and macrophages (Fig. 4). Villi in few cases were hyperplastic and elongated with cellular infiltrates. At few places, hypercellular distended villi were fused together, giving a solid appearance of mucosa. Mixed population of celular infiltrate consisted of lymphocyte, plasma cell and macrophages. Infiltration of a large number of macrophages changing to epitheloid cell, with fusion to form focal granuloma was observed (Fig. 5). In addition to macrophages, occasional infiltration of globule leukocytes was also observed (Fig. 6).

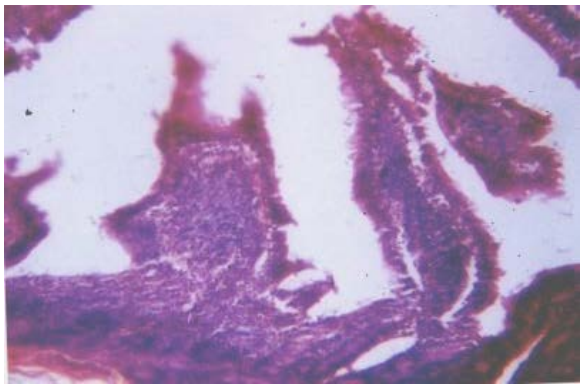

Fig. 4: Short, broad and club shaped villi with extensive cellular infiltration (H and E 25X)

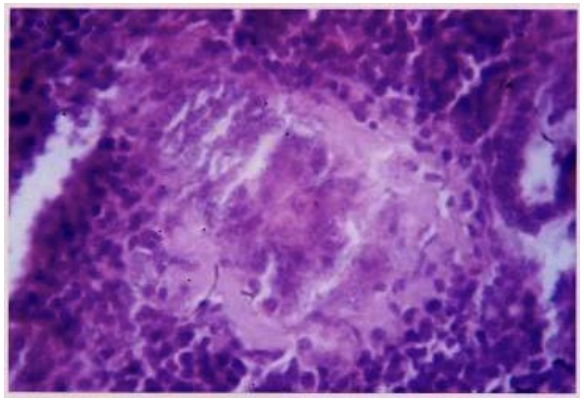

Fig. 5: Focal granuloma in the hyperplastic Peyer's patch replacing lymphoid tissues on 120 DPI (H and E 280X)

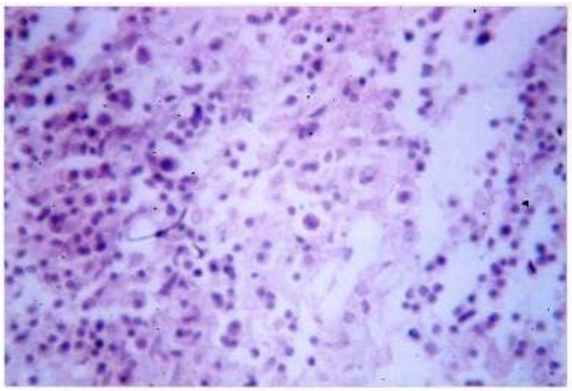

Fig. 6: Small intestine showing presence of globule leukocytes in the mucosa, predominant infiltrations of macrophages and a number of globule leukocytes on 120 DPI (H and E 280X)

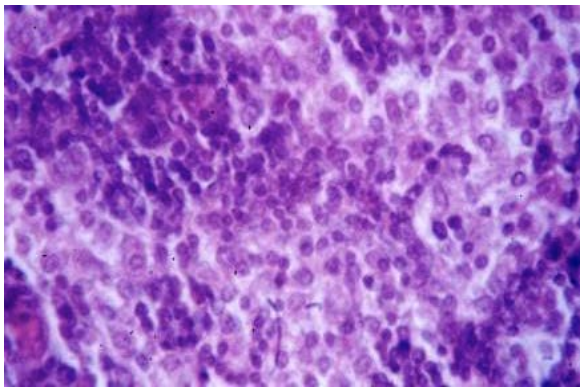

Fig. 7: Mesenteric Lymph Nodes (MLN) showing early stage of granuloma formation with the loose aggregation of macrophages on 120 DPI (H and E 280X)

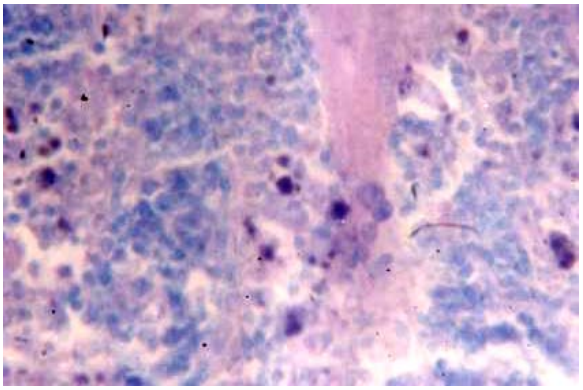

Fig. 8: Cells indistinguishable from macrophages containing acid-fast granules on 90 DPI (Ziehl-Neelsen stain 280X)

Peyer's patches were hyper plastic and lymphoid tissues were replaced by macrophages. Focal areas of granuloma composed of macrophages or epitheloid cells found in Peyer's patches. Infiltration of macrophages in the subcapsular sinuses of MLN was observed in most of the cases. Granulomas were observed in inter-follicular and peri-follicular areas of the cortex. Focal and multi focal granulomas consisting of epitheloid cells were present in the paracortex of MLN (Fig. 7).

In ZN stained tissue sections, no AFB was demonstrated in intestines and MLN of any sacrificed animal at 30 and 60 DPI. Cells indistinguishable from macrophages and containing acid fast granules were observed in intestine and MLN; although clear AFB in macrophages were not demonstrated on 90 DPI. ZN stained sections on 120 DPI revealed the AFB compatable to that of MAP in the MLN, bacilli seen as single rod and/or often clumped or studded (Fig. 8 and 9).

DISCUSSION

Studies of caprine paratuberculosis in India in post-independent era appears to be scarce and there has been little evaluation of the pathogenicity of the Indian strains of MAP. Status on early

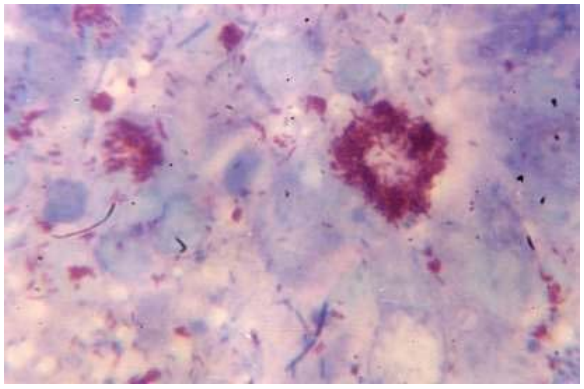

Fig. 9: Macrophages showing presence of AFB either singly or in clumps around the periphery of the cell (Ziehl-Neelsen stain 700X)

lesions of disease in experimentally infected kids by serology and pathogenecity of S-5 strain (goat's strain) of the MAP has been studied with special significance. In the present study, 52 (36.6%) faecal smear, 23 (31.08%) tissue smear for examination of AFB by direct microscopy were found to be positive in spontaneous studies. This method however is not considered to be reliable for diagnosis because it diagnoses only 1/3 of positive animals due to individual variation in the clinical stages of the disease. Clumps of bacteria are shed only in clinical stage of infection and the animal in subclinical stage eliminates only a few or no bacilli (Chiodini *et al*., 1984; Hussler and Sussex, 1997). Presence of acid fast granular cells with or without distinct AFB was perhaps due to the fact that most of the cases had early or subclinical infection and contained scarce AFB which were confirmed by histopathology, bacteriology or serology, could be due the presence of spheroplast or dead dividing bacilli within the host cell (Hussler and Sussex, 1997; OIE, 2000; Manning and Collins, 2001). Growth of organism on artificial media either from faecal or tissue samples are considered to be 100% specific for paratuberculosis (Singh *et al*., 1996). In the present study, the faecal samples and tissue samples were 26 and 17 natural cases and 6 and 8 experimental cases, respectively; were positive in bacterial culture confirming the presence of disease in goats. Diagnosis of paratuberculosis by culture method is largely hampered due to long incubation period, relatively low sensitivity and frequent contamination of cultures (Merkal *et al*., 1970; Whipple and Merkal, 1985).

For several years infections with MAP usually persist in a subclinical stage. During the subclinical period, intestinal lesions are observed which are restricted largely to the ileum and especially to the small intestinal ileocaecal valve. Hyperplasia is especially observed in the draining sites of lymph nodes that often exhibit hyperplasia with enhancement in the number of T as well as B cells as well as infiltrating macrophages. There may be additionally large number of acid-fast bacilli which are in association with infiltrating macrophages.

Immunological picture of Johne's disease mimic with that of human leprosy. In both Mycobacterioses, hyper-reactive pole, a characteristics of early stages of Paratuberculosis, there is strong cell mediated (CMI) immune reaction that limits the proliferation of the aetiological agent. This stage of JD simulates the Tuberculoid form of human leprosy (Kennedy and Benedictus, 2001; Koets *et al*., 2002). At the opposite pole, anergy occurs in the terminal stages of paratuberculosis that resembles the lepromatous form of leprosy (Merkal *et al*., 1970; Benedixen, 1978). Positive

seroreactivity was observed onwards 90 and 120 DPI at 450 nm ranging with the O.D value 0.23-0.37 (minimum) and 0.82-1.25 (maximum) in ELISA test; sera were assessed against US antigen having the cut off value of 0.235 estimated by the serum samples from very young healthy Barbari kids considered as negative for *M. avium paratuberculosis*. The specificity of ELISA was absolute in diagnosis of Paratuberculosis with no false positive result in sheep (Clarke and Little, 1996). In cattle, specificity over 95% against Paratuberculosis has been reported (Milner *et al*., 1987; Hietala, 1992). Singh (1998) reported sensitivity and specificity of ELISA as 87.09% and 86.64%, respectively, using semi purified antigen from MAP isolate harvested from a clinical cases of paratuberculosis in goat from intestinal mucosa at 0.289 cut off value. False positivity in some animals could be due to presence of cross reactive antibodies to other environmental mycobacteria or these animals might have eliminated the infection (Collins, 1996). Presence of positive response of ELISA in experimental animal with the early lesions corroborated with the findings of other workers (Shulaw *et al*., 1993; Garcia Marin *et al*., 1994; Clarke and Little, 1996; Perez *et al*., 1996). Juste *et al*. (1994) recorded similar antibody response in experimental paratuberculosis using absorbed ELISA.

Description of the early lesion has been made on the basis of composition, pattern of cellular infiltration, presence of granuloma and severity of lesions. Lesions observed on 90 DPI depicted grossly characterized mild diffused thickening and occasional folding of mucosa and predominance of lymphoid cell infiltration; observed in the early lesions of paratuberculosis in this study suggest that the animals were in beginning stage of infection as also reported in ovine paratuberculosis (Saxegaard, 1990; Perez *et al*., 1996; Kurade, 1999). Infiltration of macrophages without distinct granuloma formation was considered similar with the lepromatus type of lesions of leprosy. Distinct granuloma formation and presence of giant cells were not observed, which is in accordance with the observations of erstwhile workers (Nakamatsu *et al*., 1968; Paliwal, 1984). Early lesions observed after 3 month of infection were characterized by apparent mucosal corrugations in jejunum and ileum, enlarged and oedematus, knotted and corded MLN and conspicuous thickened lymphatic visible in the mesentery. Loose aggregation of macrophages or epitheloid cells forming focal granuloma observed in lamina propria were in accordance with reports of previous workers (Pemberton, 1979; Carbonell, 1998). The lesions envisaged simulate with that of tuberculoid form of leprosy (Lepper and Wilks, 1988). The mucosal lesions usually associated with the gut lymphoid tissue and later non lymphoid areas have been reported (Perez *et al*., 1996; Sigur-Dardottir *et al*., 2001). The distinct granuloma at places were formed and follicle became depleted after chronic out flow of lymphocytes in the lymph node, which confirmed the findings of several workers who reported similar development of lesions in paratuberculosis in sheep and goat (Rajya and Singh, 1961; Paliwal, 1984; Bernabe *et al*., 1991; Perez *et al*., 1996). Globule leucocyte responsible for hypersensitivity reaction and subsequent diarrhea has been observed; recent advances in immunology suggest that these cells could be subset of χ/δ T-cells (Tizard, 1998; Corpa *et al*., 2000). In sheep paratuberculosis, the gamma-delta T-cells were reported to play role in the presentation of lipid antigen to immune cells (Beard *et al*., 2000). Granulomatous reaction observed in MLN in goat paratuberculosis has been reported by Perez *et al*. (1996). The possibility of causing similar type of lesions by other aetiological agent was ruled out on the basis of specific pathological changes caused by the agent (Jubb *et al*., 1994; Radostits *et al*., 2000; Hajra *et al*., 2004).

CONCLUSION

Direct microscopy of faecal smear examination was considered a significant diagnostic test in herd screening of the paratuberculosis in goats. Overall 36.6% prevalence rate was recorded in the

farm goat herds in Northern India. In the experimental infection, pathogenecity of *Mycobacterium avium* subspecies *paratuberculosis* was proved with characteristics early gross and microscopic lesions and serologic response by ELISA as observed on 90 days post infection (DPI) and onwards. Lesions showing infiltration of macrophages with AFB without granuloma formation simulating lapromatous form of leprosy and typical form were found. The performances of different diagnostic tests like examination of faecal smear by direct microscopy, faecal culture, scrapping smear examination, isolation of organism from tissue, gross and histopathology and plate-ELISA test had a linear relationship among them.

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