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Pathobiological investigations in ruminants affected with gastrointestinal disorders due to paratuberculosis disease

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Abstract

The present study was carried out to investigate the pathobiology of paratuberculosis in ruminants having history of gastrointestinal tract disorders brought to the Department of Veterinary Pathology, LUVAS, Hisar, Haryana. Out of 65 ruminant (38 buffalo, 10 cattle, 5 goat and 12 sheep) carcasses examined, 15 cases were confirmed for *Mycobacterium avium subsp. paratuberculosis* including 12 bovine (8 adult and 4 calves), two cases of caprine and one case of ovine species. Confirmation of paratuberculosis was done by conventional PCR method using IS*900* primers on intestinal and mesenteric lymph nodes samples. Gross pathological studies revealed transverse folds/corrugations (60% cases), necrotic mucosa with thickening (26.7% cases) and vascular changes (13.3% cases) in intestine. Mesenteric lymph nodes showed enlargement, necrosis and induration with caseation in 80%, 13.3% and 6.7% cases respectively. Histopathology investigations in intestine revealed necro-hemorrhagic enteritis (53.3%) and granulomatous enteritis (46.7%). Mesenteric lymph nodes showed most prominent histopathological changes as granulomatous lymphadenitis (53.3%) followed by chronic lymphadenitis and hemorrhagic lymphadenitis (6.7%). Histopathological classification of paratuberculosis lesions on the basis of inflammatory cells and microgranuloma in intestinal tissue sections revealed focal (7), multifocal(1), diffuse multibacillary (1), diffuse paucibacillary (5) and diffuse intermediate (1) type lesions, while mesenteric lymph nodes showed focal (8), diffuse multibacillary (1), diffuse paucibacillary (5) and diffuse intermediate (1) type lesions. Evaluation of acid fast bacilli in MAP positive cases in intestinal and mesenteric lymph nodes sections showed positive reaction in 26.7% and 33.3% cases respectively. Immunohistochemical detection of MAP in formalin fixed intestine and mesenteric lymph nodes tissue sections using anti-*MAP* polyclonal antibody showed positive results in the cytoplasm of the macrophages, giant cells and epithelioid cells. Results of present study confirm the prevalence of paratuberculosis disease in Haryana and describe the pathobiology of this disease and corroborate that immunohistochemistry complements the histopathology for the accurate confirmatory diagnosis of paratuberculosis.

Keywords: Pathology, paratuberculosis, gastrointestinal tract disorders, ruminants, IHC

Introduction

Agriculture is the backbone of the Indian economy and animal husbandry plays a vital role in its growth. Ruminants, primarily buffalo, cattle, sheep, and goat, play an essential role in Asia's livestock sector, where they are raised for milk, meat, wool, and draught power. According to the 20th Livestock Census, cattle, buffalo, sheep, and goats account for around 36.04% (193.46 million), 20.47% (109.85 million), 13.83% (74.26 million), and 27.74% (148.88 million) of the total livestock population in India, respectively (DAHD, 2019) [1]. Hence to maximize profits from the dairy sector, additional attention must be paid to the health problems of these animals. Diseases affecting both large and small ruminants frequently result in decrease in milk production and a lower return on investment. Among ruminant gastrointestinal tract illnesses, Johne's disease is important chronic granulomatous enteritis which occurs as a result of *Mycobacterium avium subsp. paratuberculosis* (*MAP*) infection, which is an incremental, intracellular acid-fast bacterium (CLARKE, 1997) [2]. MAP affects a wide variety of animals, most notably ruminants such as cattle, buffalo, sheep and goats (SINGH *et al*., 2013) [3]. Calves under one month are most susceptible to infection; however, clinical disease does not normally appear in cattle until they are more than 2 years old, and approximately 10 to 15 percent of sick animals' exhibit clinical indications of disease (CLARKE, 1997) [1]. Granulomatous enteritis, mesenteric granulomatous lymphadenitis, and lymphangitis define the disease, with *MAP* being restricted to the small intestine and draining lymph nodes (Buergelt, 2004; Zachary and Mcgavin, 2012) [5, 4]. However, it may be disseminated to liver, uterus, fetus, milk, urine and semen. The MAP has also been linked to inflammatory bowel illness (Crohn's disease) in humans

(Hermon-Taylor, 2000) [6], implying that it has zoonotic potential.

Mycobacterium paratuberculosis detection and diagnosis are typically challenging due to the long incubation period (4 months to 15 years) and a lack of accurate tests that can anticipate infection (NIELSEN, 2008) [7]. Clinical symptoms, postmortem lesions, histology, and diagnostic procedures, including direct testing such as faecal smears, fecal culture, and polymerase chain reaction, are used to make the diagnosis. The polymerase chain reaction (PCR) assay is a more advanced technology that can detect *MAP* and identify it from other Mycobacterium species and subspecies (Chaudhary, 2009) [8]. However, there is paucity of literature on comprehensive studies related to paratuberculosis in Haryana region. In light of the above facts, thorough pathobiological investigations in ruminants with gastrointestinal tract diseases associated with paratuberculosis were carried out.

Material and Methods

The detailed study was conducted on 15 paratuberculosis positive cases (12 cattle and buffaloes, two sheep, one goat) out of total 65 ruminants carcasses (cattle, buffalo, sheep, and goat) tentatively diagnosed for gastrointestinal tract problems that were reported for a postmortem evaluation to Veterinary Pathology department at LUVAS, Hisar.

For molecular investigation, representative intestine and mesenteric lymph nodes tissue samples were collected during post-mortem examination in sterile vials and stored at -20ºC. Collected samples were screened for *MAP* by employing conventional PCR assay using self designed IS*900* specific forward and reverse primers as F(TTCTTGAAGGGTGTTCGGGG) and R(AGCCAGTAAGCAGGATCAGC), respectively which aimed to amplify a DNA fragment of 778 bp. The PCR reaction was standardized for each primer by varying the concentration of the reaction mix and cycling conditions were optimized and calculated, accordingly. The products of the PCR reactions were resolved using conventional agarose gel electrophoresis (AGE) using 1% w/v agarose (Sigma) containing ethidium bromide at 0.1 µg/µl in tris-acetate-EDTA (TAE) buffer (Thermo Scientific) along with 100 bp DNA ladder (Thermo Scientific). The DNA bands were visualized and imaged using E-gel imager system with UV light base (Thermo Scientific).

For detailed pathological investigations, post-mortem examination of all the ruminant carcasses suspected as paratuberculosis was conducted and gross pathological changes were recorded. For histopathological examination, demonstrative tissue samples from lesion specific organs were identified and preserved in 10% neutral buffered formalin. The fixed tissues were rinsed in running water, dehydrated in graded ethyl alcohol, clarified in benzene, and then embedded in paraffin wax having melting point of 60-62ºC. The standard haematoxylin and eosin (H&E) staining procedure and the modified Ziehl-Neelsen (Z-N) staining method were used to stain paraffin sections, which were cut at a thickness of 4-5µm (LUNA, 1968) [9].

Detection of *MAP* antigen in formalin fixed tissue samples of intestine and mesenteric lymphnodes was attempted using bovine polyclonal anti-*MAP* antibody (received from Department of Biotechnology, GLA University, Mathura) in cases that were found positive in molecular investigation and were showing granulomatous lesions on routine H&E staining. For IHC, 3-4 µm thick paraffin tissue sections were cut which were taken on 3-Aminopropyl-triethoxysilane (APES) coated slides. Tissue sections were deparaffinized rehydrated in graded ethanol (100%, 95%, 85%, 70% and 50%) for 1 min each at room temperature. The tissue sections were microwave-irradiated for 30 min in a Coplin jar containing 0.01M citric buffer, pH 6.0, to retrieve the antigen. The slides were placed in 3% hydrogen peroxide (H_2O_2) solution in absolute methanol for 15 min to quench the endogenous peroxidase. The non-specific sites were blocked by 5% normal goat serum (Sigma Chemicals, USA) prepared in phosphate buffered saline (PBS, pH 7.4) for 30 min. Optimal concentration of primary polyclonal antibody was determined to be 1.100 dilution using standard immunohistochemical protocol. Secondary antibody was used at 1:20 dilution in 1% BSA prepared in PBS. Coloured reactions were developed with 3-Amino-9-ethylcarbazole (AEC, Sigma Chemicals, USA) as staining substrate which was prepared following the instructions of the manufacturer which gave brick red (reddish brown) colour and Mayer's hematoxylin was used as a counterstain (Sigma Chemicals). Sections were mounted in aqueous mounting medium CC/Mount™.

Results

Out of 65 ruminants (48 bovine, 5 goat and 12 sheep) carcasses studied, 15 cases including 12 bovine, two cases of goat and one case of sheep species were positive for paratuberculosis by detecting MAP with conventional PCR assay using self designed IS*900* primers with product size of 778 bp (Fig. 1). Most of the positive cases were found in adult buffalo (7) followed by buffalo calves (3), adult cattle (1), cattle calf (1), goat (1), goat kid (1) and sheep (1). The positive control for *MAP* used in the study was obtained from field case which was confirmed with polymerase chain reaction assay and sequencing by the Department of Animal Biotechnology, LUVAS, Hisar.

The gross pathological lesions observed in paratuberculosis affected ruminant carcasses are described in Table 1. Intestine sections revealed transverse folds/corrugations in 60% cases followed by thickened necrotic mucosa (26.7%) and vascular changes with mucosal folds (13.3%). Bovine predominantly showed transverse folds/corrugations type of gross changes in intestine (Fig. 2, 3). Sheep and goat carcasses mainly revealed necrosis and thickening of mucosa. The main gross lesions observed in mesenteric lymph nodes was enlargement with vascular changes (80% cases) followed by enlargement with necrotic foci (13.3% cases) and induration with caseation (6.7% cases). In bovine, mesenteric lymphnodes appeared reddish and enlarged (Fig. 4) in most of the cases. Necrosis and caseative type lesions (Fig. 5) were observed in mainly sheep and goat. Gross lesions observed in other visceral organs were vascular changes in forestomach, abomasum, heart, liver, lungs and spleen. Lungs in few cases also revealed pneumonic lesions while kidneys appeared shrunken atrophied in many cases. Suppurative changes were also observed in liver and spleen in cases with mixed infections of suppurative bacteria.

The histopathological lesions observed in paratuberculosis

affected ruminants are described in Table 2. The results revealed that in intestine main lesion observed was necro-hemorrhagic enteritis (53.3%) followed by granulomatous enteritis (46.7%). Mesenteric lymph nodes showed most prominent histopathological changes as granulomatous lymphadenitis (53.3%) followed by chronic lymphadenitis (40.0%) and hemorrhagic lymphadenitis (6.7%). Granulomatous lesions predominate in bovine carcasses. Further classification of histopathological lesions carried out on the basis of infiltration of inflammatory cell types, presence of microgranuloma and presence of acid-fast bacterium in mesenteric lymph nodes and intestine is given in Table no. 3. The results revealed that the main lesion type observed in mesenteric lymph nodes in ruminants was focal type (7), followed by diffuse paucibacillary (5) and multifocal (1) types. In case of intestine, most frequently observed lesion was focal type lesion (8) followed by diffuse paucibacillary, diffuse multibacillary and diffuse intermediate type lesions. Evaluation of acid-fast bacilli amount according to the lesional types in intestinal and mesenteric lymph node tissue sections revealed acid fast positivity in 4 (26.7%) and 5(33.3%) cases, respectively (Table 4,5).

In focal type lesions there was formation of small granulomas due to infiltration of macrophages having vesicular nuclei and abundant foamy cytoplasm (Fig. 6). The multinucleated giant cells were also identified in the lesions at isolated areas focally with or without fibrous tissue proliferation surrounding the granuloma.

Multifocal lesions were most commonly found at the tips of the villi, creating a mild thickening. but do not modify the normal architecture. Diffuse multibacillary lesions were characterized by diffuse infiltration of epithelioid cells, macrophages, lymphocytes and giant cells in the lamina propria, causing significant mucosal thickening. Because of the infiltration, the glands were widely separated, and the villi were frequently joined. Granulomas were observed primarily in the middle part of villi (Fig.7) and medullary portions of lymph node (Fig.8). Acid fast staining confirmed pink coloured Mycobacterium in the cytoplasm of macrophages, epithelioid cells and gaint cells (chronic inflammatory cells) which are infiltrated in intestinal mucosa, sub mucosa and mesenteric lymph nodes (Fig.9). Diffuse intermediate type granulomatous enteritis was characterized by moderate infiltration with macrophages, lymphocytes and giant cells in lamina propria of mucosal folds (Fig.10). Diffuse lymphocytic (paucibacillary) lesions were characterized by severe and diffuse granulomatous inflammation due to infiltration of mainly lymphocytes with few macrophages and multinucleated giant cells (Fig.11). Evaluation of acid fast bacilli in *MAP* positive cases in intestinal and mesenteric lymph nodes sections showed positive reaction in 26.7% and 33.3% cases respectively mostly in the diffuse paucibacillary type of lesions.

Lesions noticed in other visceral organs in paratuberculosis affected carcasses revealed rumenitis, reticulitis and omasitis with congested blood vessels along with infiltration of leucocytes in mucosal epithelium. There was abomasitis with goblet cell hyperplasia, congestion and hemorrhages along with marked infiltration of mononuclear cells and polymorphonuclear cells in the mucosa. Most carcasses revealed telangiectasis, perivascular reaction, congestion and hemorrhages, fatty vacuolar degenerative changes of hepatocytes. Few cases with mixed infection also revealed focal necrotic hepatitis, suppurative perihepatitis with infiltration of the neutrophils, vacuolar degenerative changes along with centrilobular necrosis and kupffer cells hyperplasia. Kidney in many cases revealed lesions of chronic interstitial nephritis with hyaline cast, multifocal areas of infiltration of leucocytes in glomeruli, tubules and medullary areas along with thickening of capsules. There were degenerative changes in the tubular epithelial cells. Spleen showed haemosiderosis with congestion and hemorrhages in red pulp areas and necrosis with depletion of lymphocytes in white pulp areas, RE cell hyperplasia and capsular thickening. Lung sections showed serofibrinous pneumonia with congestion, hemorrhages, serous fluid and emphysema along with pleuritis in few cases.

Immunohistochemical staining was done using anti-*MAP* polyclonal antibody in formalin fixed tissue sections of intestinal wall and mesenteric lymph nodes of adult cattle that were showing granulomatous lesions on routine hematoxylin and eosin staining and also showed the presence of *MAP* in modified Ziehl-Neelsen staining. The sample was also confirmed by polymerase chain reaction assay and sequencing by the Department of Animal Biotechnology, LUVAS, Hisar. Positive IHC results were seen as brick red coloured reaction in the cytoplasm of the giant cells, macrophages and epithelioid cells indicating presence of MAP (Fig. 12, 13). This indicated the usefulness of IHC in diagnosis of *MAP* in clinical and subclinical types.

Discussion

The diagnosis of paratuberculosis has been made easier by the identification of the MAP-specific genetic element IS900 and the application of PCR-based methods. Earlier researchers also have confirmed JD in Jamunapari goat by extracting DNA from faecal samples and targeting IS*900* gene sequence of *MAP* (GREEN *et al*., 1968; KANIMOZHI *et al*., 2017) [11, 10] and have also confirmed in their studies that *MAP* gets principally confined to small intestine and draining lymph nodes. Hence samples from intestine and mesenteric lymphnodes could be used for diagnosis of MAP (Buergelt *et al*., 2004) [5]. Significance of paratuberculosis disease diagnosis is important due to its association with similar human diseases and economic effects related to it. Previous workers has reported isolation of *MAP* from diseased persons with inflammatory bowel syndrome (Crohn's disease) suggesting a zoonotic potential of this organism (SCANU *et al*., 2007; HERMON-TAYLOR *et al*., 2000) [12, 6]. Many workers have advised upgrading JD control programmes and screening animals at the farm level using sensitive testing like PCR due to its necessity (Buergelt *et al*., 2004) [5].

Pathologically similar gross findings in cattle (Buergelt *et al*., 1978) and goats (Singh *et al*., 2017) [14] have also been described by earlier workers such as enlargement of the mesenteric lymph nodes in the initial stages of an infection and mucosal thickening and corrugations of ileal mucosae, enlargement with oedema of the mesenteric lymph nodes, dilatation of the lymphatics in later stages of the infection. Previous researchers also reported pathology of paucibacillary paratuberculosis in goats and revealed mild to moderate transverse corrugations of distal ileum and

junction of ileo-caecal part of the intestine, enlargement of mesenteric lymph nodes by 2-3 times the normal size and loss of distinction between cortex and medulla due to appearance of onion-like concentric caseation (Sivaseelan *et al*., 2011) [15].

Based on the amount and pattern of cellular infiltration, previous researchers it was observed that in natural cases of paratuberculosis in goats the lesions can be focal type, diffuse multibacillary type, diffuse lymphocytic type or diffuse mixed type (CORPA *et al*., 2000). Similar results were reported in previous studies in case of bovine and ovine where more or less focal lesions were found in intestinal as well as lymph node tissues (Perez *et al*., 2005) [17] and in intestinal lymphoid tissues (Gonzalez *et al*., 2005) [18]. Focal lesions in the primary stages of the *MAP* infection suggest representing initial forms of the disease (Kurade, 2004) [19]. Scientists who observe same type of lesions were also of the view that focal lesions indicate persistent infection during early life which develops due to latency of bacteria often limited by the immune responses (Perez *et al*., 1996; Corpa *et al*., 2000) [20, 16]. According to few workers multifocal lesions based on degree of leucocytic infiltration may be graded as mild and severe forms in bovine species (Buergelt *et al*., 1978) [13]. Histopathologically in diffuse multibacillary lesions, researchers observed that there was dilation of intestinal glands which was most probably associated with the presence of infiltrate in the lamina propria causing its occlusion (Gelberg *et al*., 2001; Gonzalez *et al*., 2005) [21, 18]. Diffuse lymphocytic (paucibacillary) lesions were found in five cases in our study. Similar findings regarding the diffuse type lesions were also reported earlier where it was found that the main inflammatory cells were lymphocytes. Besides the lymphocytes, well-differentiated Langhan's type of giant cells and macrophages were also reported (Gonzalez *et al*., 2005) [18]. Diffuse intermediate lesions were reported and described by other researchers (Gonzalez *et al*., 2005; Perez *et al*., 1996) [18, 20]. In caprine paratuberculosis, lesions can be distinguished as the paucibacillary or borderline tuberculoid form which showed infiltration of lymphocytes along with macrophages having few bacteria, while multibacillary or borderline lepromatous form had infiltration of large number of bacteria containing macrophages (Reddy *et al*., 2012) [22]. It was postulated that macrophages containing MAP cross the basal membrane and move to mucosa for the passive shedding of bacteria as histologically acid-fast bacilli-containing macrophages can be detected between the epithelial cells and in the intestinal lumen (Momotani *et al*., 1988) [23]. In support of results of present study, previous researchers have also reported the presence of few acid-fast bacilli in the granulomas or giant cells in the ZN staining (Perez *et al*. 1996; Gonzalez *et al*., 2005) [20, 18].

According to some research studies, immunohistochemical methods may be more sensitive and specific than ZN acid-fast staining since they may identify modified microorganisms with damaged cell walls, soluble MAP, and cell Fragments in addition to whole, living organisms (Plante *et al*., 1996) [24]. Comparison studies of IHC and acid-fast testing for MAP revealed comparable sensitivity of these tests without any significant differences, but the sensitivity of these two tests was found to be less than the culture method (MARTINSON *et al*., 2008) [25]. However on contrary, researchers have also reported positive IHC reaction in 29 out of 32 animals that were positive by culture or IS*900* probe (Brees, 2000) [26]. It was concluded that immunohistochemistry is more sensitive and specific than acid-fast staining and could be adopted as quick diagnostic method (Brees, 2000) [26]. The results of present study also showed the positive utility of IHC in detection of bacterial antigens in the tissue sections which could be applied for rectal pinch tissue sections for diagnosis of sub clinical cases of paratuberculosis which usually remain undiagnosed.

Although, IHC method has good sensitivity but the limitation of cross reactivity of *MAP* with other *Mycobacterium* species such as *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Mycobacterium leprae* reduces the specificity. However for the confirmation of the paratuberculosis previous workers have found variable results for different diagnostic techniques. In agreement to our observations previous workers also observed that subclinical disease diagnosis is more difficult by histopathology alone as lesions may be subtle and organisms may be rare (Buergelt and GINN, 2000) [27].

Table 1: Gross lesions observed in ruminants affected with paratuberculosis disease

Organs	Gross lesions	Animals species			Total	Percentage (%)
		Bovine	Ovine	Caprine		
	Transverse folds/corrugations	9	0	0	9	60
Intestine	Thickened necrotic mucosa	1	1	2	4	26.7
	Vascular changes with mucosal folds	2	0	0	2	13.3
	Total	12	1	2	15	
Mesenteric lymph nodes	Enlargement with vascular changes	12	0	0	12	80.0
	Enlargement with necrotic foci	0	0	2	2	13.3
	Induration and caseation	0	1	0	1	6.7
	Total	12	1	2	15	

Table 2: Histopathological lesions observed in ruminants affected with paratuberculosis

Organs	Lesions	Animal species			Total	Percentage (%)
		Bovine	Ovine	Caprine		
Intestine	Necro-hemorrhagic enteritis	6	0	2	8	53.3
	Granulomatous enteritis	6	1	0	7	46.7
	Total	12	1	2	15	
Mesenteric lymph nodes	Hemorrhagic lymphadenitis	1	0	0	1	6.7
	Chronic lymphadenitis	5	0	1	6	40.0
	Granulomatous lymphadenitis	6	1	1	8	53.3
	Total	12	1	2	15	

Table 3: Classification of the histopathological lesions in paratuberculosis affected carcasses (n=15)

Type of lesion	Number of cases identified in ruminants							
	Mesenteric lymph nodes			Total	Intestine			
	Bovine	Caprine	Ovine		Bovine	Caprine	Ovine	Total
Focal	7	0	0	7	8	0	0	8
Multifocal	1	0	0	1	0	0	0	0
Diffuse multibacillary	1	0	0	1	1	0	0	1
Diffuse paucibacillary	2	2	1	5	2	2	1	5
Diffuse intermediate	1	0	0	1	1	0	0	1
Total	12	2	1	15	12	2	1	15

Table 4: Evaluation of Acid-fast bacilli amount according to the lesional types in intestinal tissue sections of ruminant carcasses (n=15)

Type of lesions in Intestinal tissue sections	Numbers of cases identified in ruminants				Amount of acid-fast bacilli by Ziehl-Neelsen staining in ruminants			Total no. of ZN stained sections	Percentage (%)
	Bovine	Caprine	Ovine	Total	Bovine	Caprine	Ovine		
Focal	8	Nil	Nil	8	0(8)	Nil	Nil	0(8)	0
Multifocal	Nil	Nil	Nil	0	Nil	Nil	Nil	0	0
Diffuse multibacillary	1	Nil	Nil	1	+++(1)	Nil	Nil	1(1)	100
Diffuse paucibacillary	2	2	1	5	+(2)	0(2)	0(1)	2(5)	40
Diffuse intermediate	1	Nil	Nil	1	+(1)	Nil	Nil	1(1)	100
Total	12	2	1	15	4(12)	0(2)	0(1)	4(15)	26.7

Table 5: Evaluation of Acid-fast bacilli amount according to the lesional types in mesenteric lymph nodes sections of ruminant carcasses (n=15)

Type of lesions in mesenteric lymph nodes	Numbers of sections examined in ruminants			Total	Amount of acid-fast bacilli by Ziehl-Neelsen staining in ruminants			Total no of ZN stained sections	Percentage (%)
	Bovine	Caprine	Ovine		Bovine	Caprine	Ovine		
Focal	7	Nil	Nil	7	0(7)	Nil	Nil	0(7)	0
Multifocal	1	Nil	Nil	1	+(1)	Nil	Nil	1(1)	100
Diffuse Multibacillary	1	Nil	Nil	1	+++(1)	Nil	Nil	1(1)	100
Diffuse paucibacillary	2	2	1	5	+(2)	0(2)	0(1)	2(5)	40
Diffuse intermediate	1	Nil	Nil	1	+(1)	Nil	Nil	1(1)	100
Total	12	2	1	15	5(12)	0(2)	0(1)	5(15)	33.3

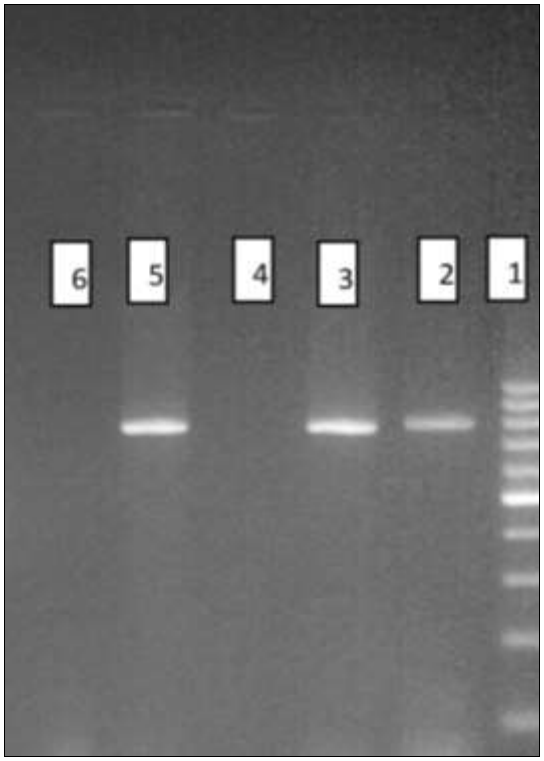

Fig 1: Agarose Gel Electrophoresis of PCR amplified products for MAP showing Lane-1: 100 bp ladder, Lane-2, 3: Unknown samples detected positive for *Mycobacterium avium ssp. paratuberculosis* (*MAP*), Lane-4: Negative control, Lane-5: Positive control of *MAP*), Lane-6: Unknown sample detected negative for *MAP*)

Fig 2: Intestine showing reddish mucosa with transverse mucosal folds (Adult buffalo)

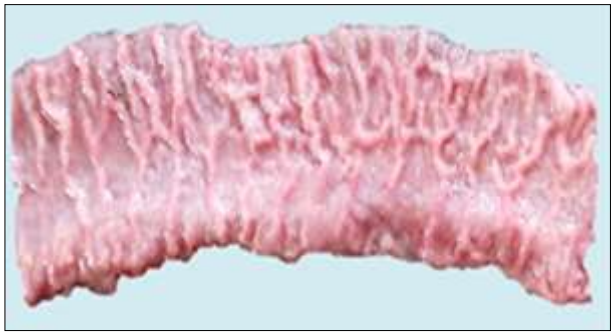

Fig 3: Intestine with prominent transverse corrugations (Buffalo calf)

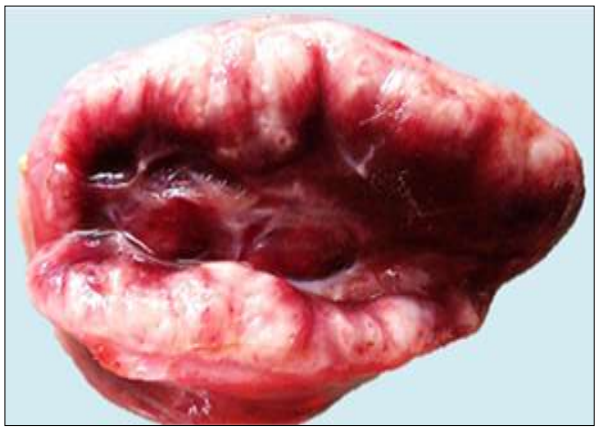

Fig 4: Cut section of enlarged mesenteric lymph nodes showing edema, redness and hemorrhages (Adult cattle)

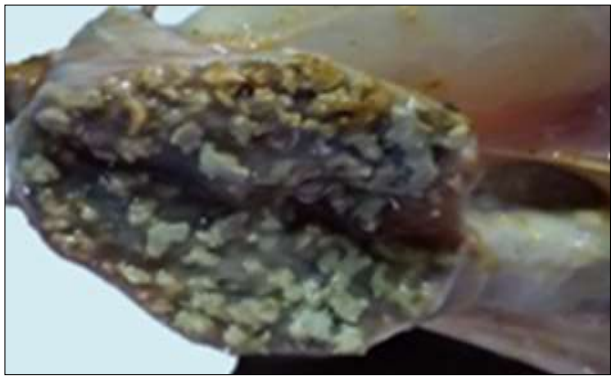

Fig 5: Mesenteric lymph nodes showing caseation and necrosis (Sheep)

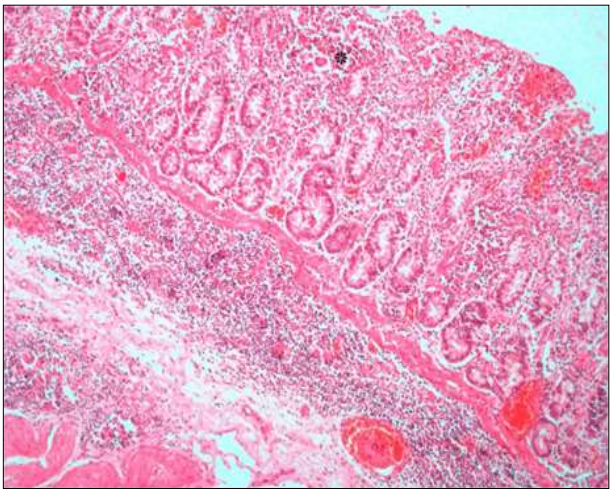

Fig 6: Focal enteritis characterized small granulomas (asterisk) formed by infiltration of macrophages, epithelioid cells and lymphocytes (H&E 200X; Adult buffalo)

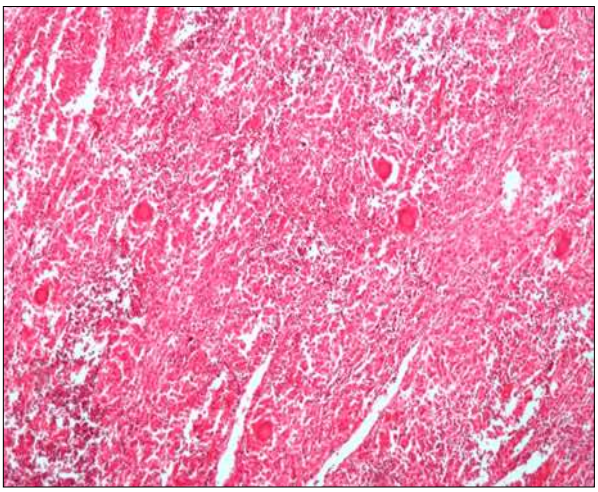

Fig 7: Diffuse multibacillary granulomatous enteritis characterized by thickening of mucosa with infiltration of macrophages, epithelioid cells in mucosa and giant cells (H&E 200X; Adult cattle)

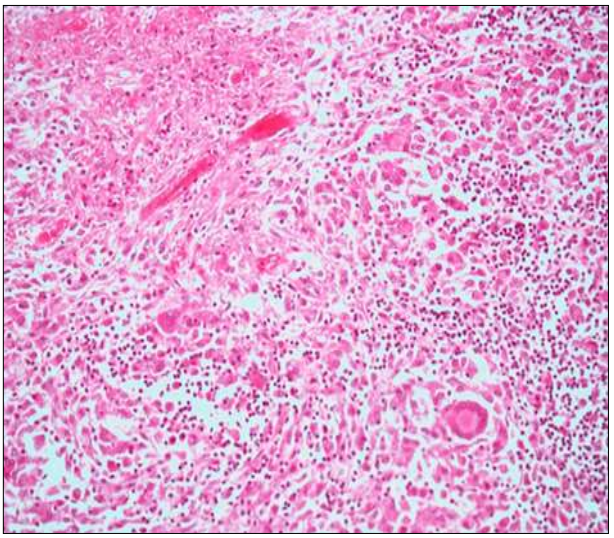

Fig 8: Diffuse multibacillary granulomatous lymphadenitis characterized by caseative necrosis and infiltration of Langhan's type of giant cells (arrow) and macrophages (H&E 100X; Adult cattle

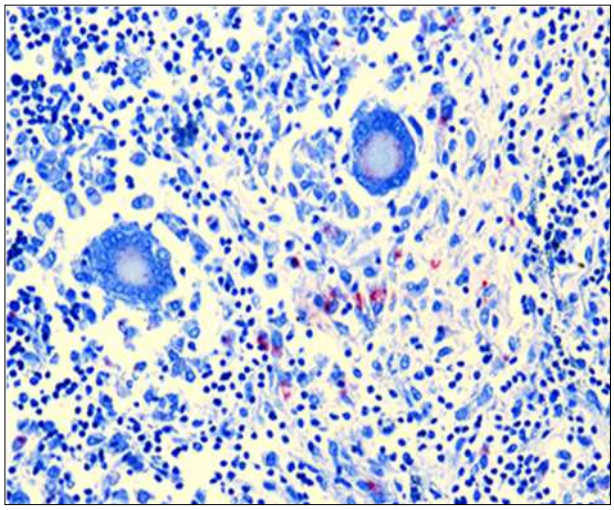

Fig 9: Diffuse multibacillary granulomatous lymphadenitis showing pink coloured acid fast bacilli in cytoplasm of macrophages (asterisk) and Langhan's type of giant cells (ZN stain, 400X; Adult cattle)

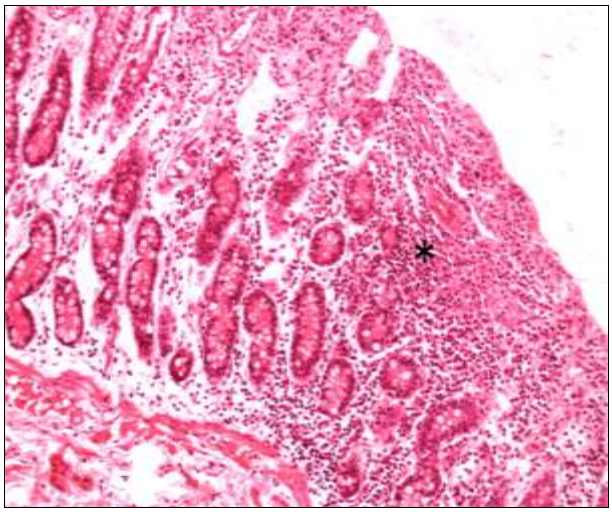

Fig 10: Diffuse intermediate granulomatous enteritis with lymphocytic, macrophages and giant cells infiltration in intestinal mucosa (asterisk) (H&E 200X; Adult buffalo)

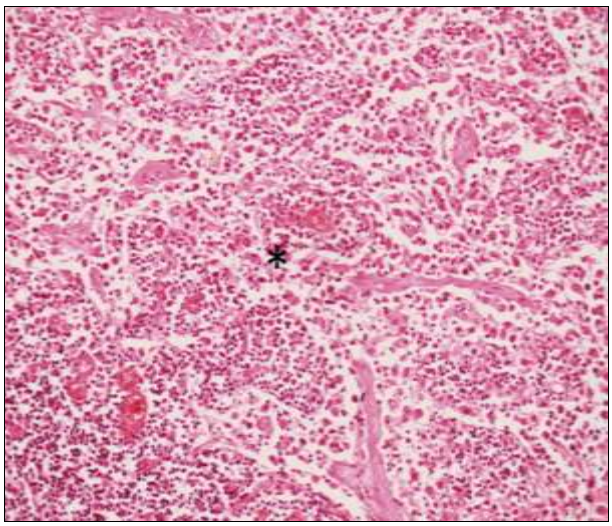

Fig 11: Diffuse paucibacillary lymphadenitis with infiltration of macrophages, epithelioid cells and lymphocytes (asterisk) in medullary sinuses (H&E 200X; Goat)

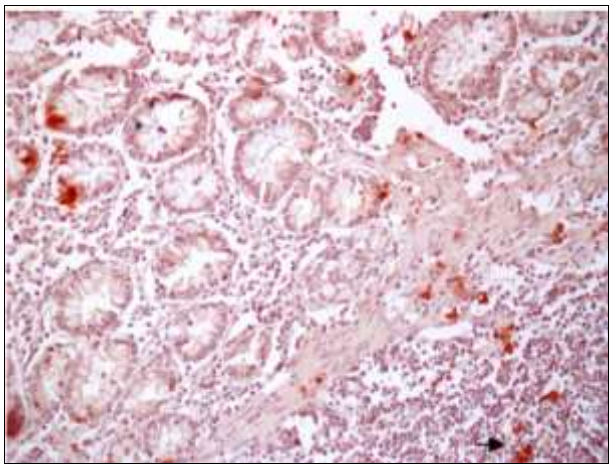

Fig 12: Intestine showing brick red coloured positive immunostaining (arrow) in cytoplasm of macrophages in mucosa and sub mucosa (IHC 200X; Adult cattle, *MAP*)

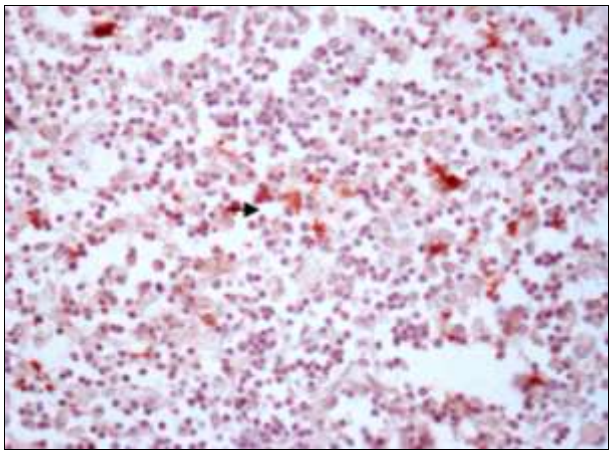

Fig 13: Mesenteric lymph nodes showing brick red coloured positive immunostaining in cytoplasm of macrophages (IHC400X; Adult cattle)

Conclusions

Results of present study confirm the prevalence of paratuberculosis disease in Haryana and corroborate that IHC using polyclonal antibodies complements the diagnosis of subclinical paratuberculosis; but combined application of more than one test are required to complement the accurate histopathological diagnosis.

Conflicts of interest/Competing interests

The authors declare no conflict of interest related to this article.

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Author Contributions

Dr Subhash, Dr Deepika: Conceptualization, Methodology, Investigation, Project administration, Data Curation, Formal Analysis, Writing- Original draft, Dr Vikas Nehra: Conceptualization, Pathological methodology, Dr Renu Gupta: Molecular methodology, Dr Shoorvir Singh: Resources for IHC studies, Dr RP Gupta and Dr Gulshan Narang: Conceptualization, Funding acquisition, Supervision for study as Principal investigator of RKVY scheme running in the Department of Veterinary Pathology and performed the final review of the manuscript.

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