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Paratuberculosis in different breeds of sheep: A retrospective study of cases

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ABSTRACT

Spontaneous ovine paratuberculosis in an organized farm was diagnosed based on histopathological lesions, demonstration of acid-fast bacilli in different visceral organs, and detection of antibody levels against *Mycobacterium paratuberculosis* by agar gel immunodiffusion test (AGID). Out of 190 morbid specimens examined histopathologically, 77% of specimens had pathological lesions, which included predominant epithelioid cell formation, infiltration of lymphocytes, monocytes and macrophages. In acid-fast staining of tissue sections, 74% of intestinal specimens and 53% of mesenteric lymph nodes were positive for the presence of acid-fast bacilli. Hematologically, the animals were showing leucopenia, macrocytic and normochromic anemia. Out of 49 animals tested, 28.5% of animals were positive for antibodies against *M. paratuberculosis*. Local breeds, namely Nilagiri and Sandyno sheep, were found to be more susceptible than exotic breeds, namely Dorset. Females were more susceptible than males. Mortality was noticed in the age group of 4 months to 10 years.

Introduction

Paratuberculosis in sheep is characterized by chronic granulomatous enteritis, regional lymphangitis and lymphadenitis leading to progressive loss of body weight [1]. The disease is caused by *Mycobacterium avium*, subspecies *paratuberculosis* (*Mycobacterium paratuberculosis*). Diagnosis of the disease is difficult based on clinical signs alone as only limited animals may have diarrhea [2] and which may be due to several factors. Demonstration of acid-fast bacteria in feces is less sensitive [3,4] and delayed hypersensitivity reaction may simply indicate exposure and the presence of stimulated memory cells [5]. Serological tests like agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay have been reported as highly sensitive tests [6–8]. The present paper describes the diagnosis of ovine paratuberculosis in an organized farm based on histopathology of different organs, demonstration of acid-fast bacteria in tissue sections, hematology and AGID test.

Materials and methods

History

This study is based on an organized farm consisting of 1100 sheep, including lambs, belonging to the Dorset, Sandyno and Nilagiri breeds and their mixes. Their age range is from below one month to 12 years. The males and females were housed separately on the farm. Young animals were allowed to mix and graze with adults during the day in the vicinity

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of the farm premises. Sporadic mortality with loss of body condition and diarrhea was reported. The affected sheep were treated with antibiotics; although the condition of enteritis improved, it reappeared shortly and became chronic enteritis. An investigation was undertaken to find out the cause of mortality.

Samples

Thirty-three heparinized blood and 49 sera samples from ailing animals were collected randomly for hematological and serological tests; 190 morbid specimens consisting of liver, spleen, kidney, heart, lung, intestine and mesenteric lymph node from dead animals preserved in a 10% formalin solution were received over a period of approximately 2 years for histopathological studies.

Laboratory examination

Hemogram of blood sample was carried out following standard hematological procedures [9]. Morbid specimens were routinely processed, embedded in paraffin, sectioned at 5 μm thickness and stained by Hematoxylin and Eosin (H&E) method. Unstained paraffin embedded tissue sections were stained following the Ziehl–Neelson (ZN) method.

A scoring was adopted to assess the severity of the microscopical lesions in the intestine and mesenteric lymph nodes as mild (1+); moderate (2+); severe (3+) and negative (0) for both H&E stained and ZN-stained tissue sections as described by Michael and Bastinello [10].

AGID: The test was done on microscopic slides (75 mm × 25 mm) loaded with 4 ml of 1% molten agarose (low EEO). The antigen was charged in central wells and sera in peripheral wells at a volume of 20 μl. The diameter of the wells and the distance between the wells were 4 mm. The slides were kept at room temperature in a humidified box, and results were read at 24, 48 and 72 h. Positive sera were assessed for antibody levels by the quantity AGID (QAGID) test. The antigen for the AGID test was kindly supplied by Dr. B.N. Tripathi, Division of Pathology, Indian Veterinary Research Institute, UP, India.

Polymerase Chain Reaction (PCR): Representative samples of intestine were subjected to PCR analysis to confirm acid-fast bacteria. DNA was extracted using DNeasy tissue kit (Cat. No. 69504). A single step PCR was carried out based on the method of Giese and Athrens [11] PCR mixture consisting of 2×Red dye PCR master mix 12.5 μl; 1 μl (30 pmol) of forward primer #11; 5′-GTCGTTAATAACAATGCAG-3′ and 1 μl (30 pmol) reverse primer #36; 5′-GGCCGTGCGTTAGGCTTCGA-3′; template 1 μl (50 ng); distilled water 9.5 μl.

The PCR master mix was mixed with the PCR primers (forward and reverse), template, DNA and amplified with initial denaturation for 3 min at 94 °C and denaturation for 1 min at 94 °C followed by annealing for 1 min at 55 °C and extension for 1 min at 72 °C. Final elongation was carried out for 7 min at 72 °C. Denaturation, annealing and extension were repeated for 35 cycles in the thermal cycler. The amplified products were separated by agarose gel electrophoresis on a 1.5% agarose gel. Agarose gel (1.5%) was prepared in IX TBE buffer containing 250 μg ethidium bromide and was poured onto the agarose gel electrophoresis apparatus (Broviga, India). Ten microlitres of the PCR product was mixed with 4 microlitres of 6× gel loading buffer and loaded into preformed wells of agarose gel. Two μl of 100 bp molecular weight ladder (Genei Pvt. Ltd., India) was also loaded in the gel. Submarine gel electrophoresis was carried at 75 V for 1 h. The gel was viewed under UV gel electrophoresis system (Vilber Lourmat, France) and documented.

Results

Clinical signs and mortality

Clinical signs of emaciation and diarrhea were recorded in ailing animals. Mortality was mostly in the age group of 4 months to 10 years (Table 1). Mortality was sporadic and spread throughout the year. Pneumonia, enteritis and debility were the major causes of mortality. Mortality was high in Nilagiri breeds followed by Sandyno, Dorset–Nilagiri mix, Sandyno–Nilagiri mix and Dorset, respectively (Table 1).

Hematology

Mean hemogram of blood collected from 33 ailing animals were as follows: Hbg% – 7.147 ± 0.292, ESR mm/h – 0.5 ± 0.153, PCV% – 33.85 ± 1.12, RBC million/cmm – 4.96 ± 0.133, WBC/cmm – 5610 ± 241.17, MCV – 68.24 ± 2.08 fl, MCHC – 21.57 ± 0.79 g/dl% and MCH – 14.72 ± 0.635 pg.

AGID

Out of 49 animals tested, 14 animals were positive and antibody titer ranged from 2^1 to 2^5 with geometric mean AGID titer of 2.35 ± 0.43 log 2 (Table 2).

Histopathology

Intestine

Histopathologically, three types of lesions were noticed. In mild lesions, mucosa was involved with focal aggregation of epithelioid cell nests usually small and indistinct, high infiltration of lymphocytes in the lamina propria (1+). In moderate lesions several distinct epithelioid cell nests of multifocal expanses of epithelioid cells were seen in mucosa and submucosa (+2); severe lesions involved all layers of the intestine with heavy infiltration of epithelioid cells in the mucosa, atrophy and clubbing of villi; obliteration of crypts of LiberKuhn. In some crypts accumulation of cellular debris was noticed. There were variable infiltration of lymphocytes, monocytes, plasma cells and macrophages in mucosa and more extensively in submucosa (3+) (Fig. 1). Neutrophils were rare. Eosinophils and lymphocytes were predominant in young animals showing mild lesions. Mild lesions (1+) were noticed in 24 cases; 71 cases recorded moderate lesions (2+); and 75 cases revealed severe lesions (3+). No lesions were recorded in 20 specimens.

A large number of acid-fast bacteria typical of *M. paratuberculosis* were seen within the macrophages/epithelioid cells or scattered in clumps in necrosed areas of mucosa and submu-

Table 1 – Mortality pattern in sheep based on breed, age and sex.

Years	Age														
	Nilagiri			Sandyno			Dorset × Nilagiri			Sandyno × Nilagiri			Dorset		
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
>1	5	0	5	0	5	5	3	4	7	0	3	3	0	0	0
>2	3	12	15	3	7	10	1	4	5	0	0	0	0	0	0
2–5	3	26	29	1	10	11	0	8	8	0	0	0	0	3	3
<5	1	14	15	0	13	13	0	3	3	0	4	4	0	0	0
Total	12	52	64	4	35	39	4	19	23	0	7	7	0	3	3

M – male; F – female; T – Total. Data not available for 54 animals.

Table 2 – Antibody titer of different sera samples by Q-AGID Test

S. No.	Titer	No. of samples
1	2^1	5
2	2^2	3
3	2^3	3
4	2^4	2
5	2^5	1
Mean value		2.35 ± 0.35

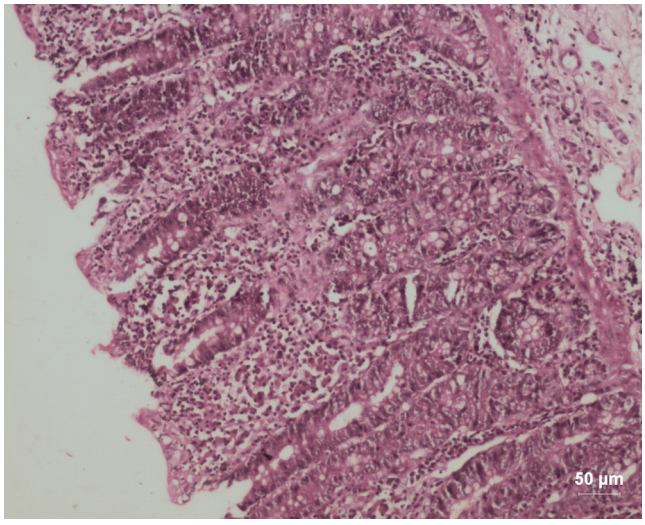

Fig. 1 – Ovine paratuberculosis – ileum – severe lesions involved all layers of the intestine with heavy infiltration of epithelioid cells in the mucosa, atrophy and clubbing of villi; variable infiltration of lymphocytes, monocytes, plasma cells and macrophages in mucosa and more extensively in sub-mucosa (3+) – H&E – Bar 10 µm.

cosa of the intestine. When there was extensive infiltration of lymphocytes with mild to moderate epithelioid cells, only a few organisms were detected. Presence of 1 to 2 organisms in a few cells was scored as 1+ (45 cases); 2–10 organisms in one cell or in scattered cells in several nests were scored as 2+ (42 cases). In heavy infiltration of epithelioid cells, a large number of acid-fast organisms were seen packed in epithelioid cells, under low power (100×) magnification (3+) (54 cases) (Fig. 2). No acid-fast organisms were detected (0) in 49 cases.

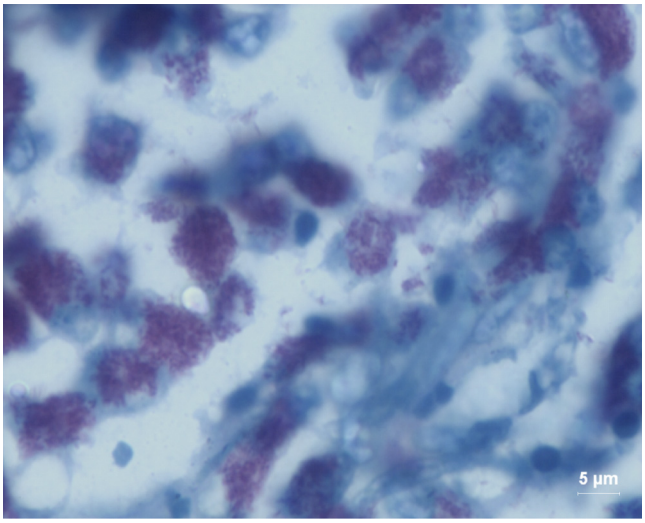

Fig. 2 – Fig. 1 Ovine paratuberculosis – ileum – large number of acid-fast organisms within epithelioid cells – Ziehl–Neelson – Bar 5 µm.

Mesenteric lymph node

Generally hyperplastic with granulomatous lesions involving cortex and medulla. Histiocytic infiltration in the medulla was seen in a few cases. The presence of an indistinct single, epithelioid cell nest in the T cell area was scored as (1+) in 37 cases; several, distinct epithelioid cell nests or multifocal expanses of epithelioid cells in the T cell area were scored as 2+ in 51 cases; numerous multifocal epithelioid cell nests or diffuse expanses of epithelioid cells in the T cell areas were scored as 3+ in 14 cases and no lesions suggestive of paratuberculosis were seen in 58 cases.

Acid-fast organisms were detected within the epithelioid cells or histiocytes, usually in low numbers when compared with the intestine. The presence of 1 to 2 organisms per cell in a few epithelioid cells was scored as 1+ in 43 cases. About 2–10 organisms in scattered cells in several nests were scored as 2+ in 20 cases; numerous organisms in many epithelioid cells were scored as 3+ in 3 cases and no acid-fast organisms were detected in 140 cases.

Liver

Mild degeneration and necrosis of hepatocytes with multifocal periportal infiltration of mononuclear cells, mostly lym-

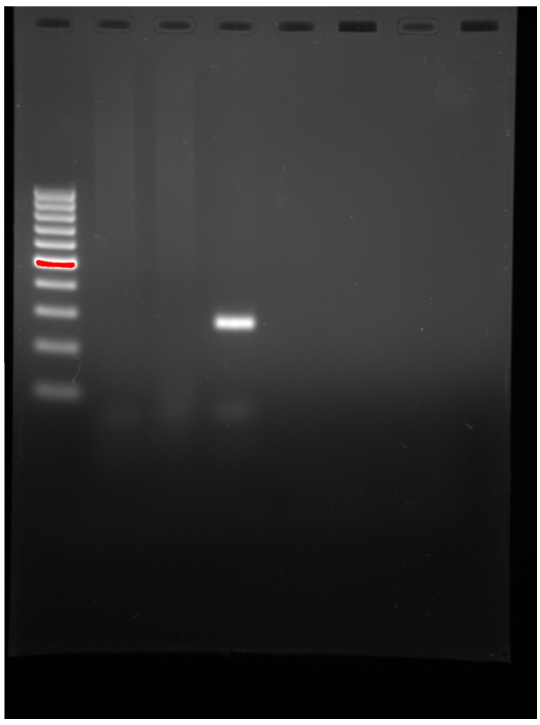

Fig. 3 – An amplicon of 277 bp showed positivity for *Mycobacterium paratuberculosis*.

phocytes, were noticed. No epithelioid cells or tubercle formations were seen.

Lungs
Chronic lobar pneumonia was recorded in 20 animals positive for paratuberculosis and suppurative bronchopneumonia seen in 2 animals positive for paratuberculosis.

Spleen
Congestion and hemosiderin pigmentation were the frequent finding.

Heart
Non-specific myocarditis was noticed in 4 animals positive for paratuberculosis lesions.

Polymerase Chain Reaction (PCR)

An amplicon of 277 bp showed positivity for *M. paratuberculosis* (Fig. 3).

Discussion

In the present study, the hemograms of ailing animals revealed leucopenia and macrocytic normochromic anemia, which was highly suggestive that the animals were suffering from a chronic wasting disease. However, the disease was diagnosed as ovine paratuberculosis by histological and serological studies.

Out of 190 morbid specimens examined histologically, 77% of the animals showed severe to moderately severe intestinal lesions and most of the animals died with clinical signs of chronic diarrhea. Predominantly epithelioid cell formation, infiltration of lymphocytes, monocytes and macrophages were observed. Similar lesions were reported earlier in the case of ovine paratuberculosis [12,13].

In acid-fast staining of intestinal sections, only 74% of specimens were positive for acid-fast bacilli. But 90% of the animals showed pathological lesions. About 53% of mesenteric lymph nodes showed pathological lesions, but acid-fast bacilli could be detected only in 34% of the specimens. About 16% of the intestinal tissues where acid-fast bacilli could not be demonstrated were showing pathological lesions suggestive of paratuberculosis. Similarly 19% of the mesenteric lymph nodes were negative for acid-fast bacilli, but positive for pathological lesions. The absence of intracellular acid-fast bacilli in the intestine and lymph nodes does not altogether remove the possibility of paratuberculosis. Since it is known that light microscopic detection of acid-fast bacilli in subclinical cases can be difficult and many factors can play a role in the failure of a pathologist to detect them [10], the mere demonstration of acid-fast bacilli also does not inconclusively indicate that the animal is suffering from paratuberculosis. However, a large number of bacilli along with pathological lesions are highly suggestive of ovine paratuberculosis.

The sensitivity of histopathological diagnosis was improved in this study since intestine and mesenteric lymph node specimens along with different vital organs were available for microscopical examination. Although lesions developed first in the gut associated lymphoid tissue following oral ingestion, similar kinds of lesions in liver, lung and lymph nodes have been described in lambs infected orally [14,15].

The common route of infections of *M. paratuberculosis* is the feco-oral route. After oral infection, the host may eliminate the organism from the intestine by intracellular killing. Cell-meditated immunity (CMI) is the principal mechanism of clearing infection. CMI may wane over a protracted period of persistent infection which leads to proliferation of bacilli and the disease with multibacillary form, whereas excessive CMI may resist killing of bacilli and encourage persistent inflammation with few or paucibacillary form [2]. In the present study, the majority of the animals were found to have the multibacillary form and a few of the animals had the paucibacillary forms.

Although the diagnosis was based on histopathological examination and demonstration of acid-fast bacteria, specific diagnosis of paratuberculosis was made by AGID test. In paucibacillary form serological tests have little significance. It is presumed that when CMI decreases, the bacilli proliferates and lyse the infected cells. The released antigen produces antibodies. High levels of antibodies are observed in advanced cases with multibacillary lesions [7,16,17]. In the present study about 28.5% of animals were positive for *M. paratuberculosis*, and possibly these animals were suffering from multibacillary infection.

The manifestation of the disease depends on several factors, which include the dose and route of infection, genetic makeup, age of the host, local and systemic immune status and environmental factors [1]. In the present study most of the affected animals were Nilagiri followed by Sandyno breeds, and the least affected breed was Dorset. Both Sandyno

and Nilagiri breeds were indigenous breeds and possibly these breeds are highly susceptible to ovine paratuberculosis.

Mortality was less in the very young and more than 5 years old animals. Possibly the young animals were protected with colostral antibodies. Another reason could be the long incubation period of the organism. In adults, the susceptibility decreases due to concomitant involution of the ileal patch and the disappearance of the favored site of persistence of the bacilli [18–20]. This study also indicated that females were more affected than males, which corroborated with the earlier work of Tripathi and Parihar [21].

Conclusion

Ovine paratuberculosis was commonly seen in sheep of the age group from 4 months to 10 years old. Nilagiri and Sandyno breeds were found to be more susceptible than the Dorset breed. Females were more susceptible than males. The intestine was mostly affected compared with other organs. Diagnosis was carried out based on pathological lesions, demonstration of acid-fast bacilli and detection of antibody levels against *M. paratuberculosis* by AGID. Acid-fast bacilli were further confirmed as *M. paratuberculosis* based on PCR.

Conflict of interest

The authors declare that there is no conflict of interest or no competing financial interest.

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