Comparative Immunology, Microbiology and Infectious Diseases 66 (2019) 101338

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Evaluation of newly developed 'six recombinant secretary proteins based 'cocktail ELISA' and 'whole cell lysate' based 'indigenous ELISA' and tissue microscopy' with 'Gold standard' histo-pathology for the diagnosis of Johne's disease in slaughtered goats and buffaloes

Kundan Kumar Chaubey[a,c], Neeraj Gangwar[b], Rajveer Singh Pawaiya[c], Gaya Prasad Jatav[d], Jagdip Singh Sohal[e], Shoor Vir Singh[a,c,*], Manju Singh[a,c,e], Saurabh Gupta[a,c], Gururaj Kumaresan[c], Naveen Kumar[f], Sujata Jayaraman[e]

[a] Department of Biotechnology, GLA University, Mathura, Uttar Pradesh, 281406, India
[b] Department of Pathology, Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya evam Go Anusandhan Sansthan, Mathura, Uttar Pradesh, India
[c] Animal Health Division, Central Institute for Research on Goats, Mathura, Uttar Pradesh, India
[d] Department of Pathology, Veterinary College, Mhow, Indore, Madhya Pradesh, India
[e] Amity Institute of Microbial Technology, Amity University, Jaipur, Rajasthan, India
[f] National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equines, Hisar, India

ARTICLE INFO

Keywords:
Mycobacterium avium subspecies *paratuberculosis*
Histo-pathology
Tissue microscopy
H&E staining
i_ELISA
c_ELISA
Johne's disease

ABSTRACT

Three screening tests {(newly developed, six recombinant secretory proteins based 'cocktail ELISA', in-house robust 'indigenous ELISA' based on semi-purified protoplasmic antigens and tissue microscopy were evaluated with 'Gold standard', histo-pathology for the diagnosis of Johne's disease in goats and buffaloes. Serum and tissues {mesenteric lymph nodes and intestines) were driven from farmer's goats (n = 77) and buffaloes (n = 40) slaughtered for harvesting meat and farm goats (n = 77), died and necropsied. Twenty seven (35%) goats and 23 (57.5%) buffaloes were positive in all the four tests. Of 134 tissues screened by histo-pathology, 79.8% MLN and 76.8%, intestines, were positive for MAP infection. In tissue microscopy, 55.2 and 52.3%, goats and buffaloes were positive, respectively. Of 117 sera screened by i_ELISA, 58.4 and 70.0%, goats and buffaloes were positive, respectively. Whereas, c_ELISA detected 55.8 and 62.5%, goats and buffaloes, positives, respectively. Twelve tissues (70.5%) of goats necropsied were positive, both in tissue microscopy and histo-pathology. 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 histo-pathology grade III and IV lesions were significantly low as compared to grade I and II. Of the four tests used for screening 268 samples, histo-pathology was most sensitive (78.3%), followed by i_ELISA (62.3%), c_ELISA (58.9%) and tissue microscopy (58.9%). Between two ELISA tests, c_ELISA using six recombinants secretory proteins, had higher specificity as compared to i_ELISA.

1. Introduction

Johne's disease (JD) cause by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is characterized by chronic granulomatous enteritis and lymphadenitis, leading to diarrhoea, progressive weight loss, reduce milk production and fertility in domestic livestock [1–3]. MAP has also been associated with number of health disorders of human beings, primates and wild animals [4–6]. JD causes huge negative impact on the livestock economy globally [7–10].Losses result from increase in cost of veterinary care, reduced milk yield, loss of productivity, short life span, long calving intervals, premature culling and increased incidence of other diseases [9–12]. Diagnosis of MAP is challenging due to non-specific symptoms, long incubation period, long or variable sub-clinical stage and shedding (intermittent or continuous) of the bacilli in faeces [13,14].

Microscopy being simple, easy to perform, quick, inexpensive and preferred as 'screening test' was evaluated with histo-pathology. Faeces are major source of dissemination of disease and due to ease of

* Corresponding author.
E-mail addresses: kundan.chaubey@gla.ac.in (K.K. Chaubey), shoorvir.singh@gla.ac.in (S.V. Singh).

https://doi.org/10.1016/j.cimid.2019.101338
Received 19 February 2019; Received in revised form 7 July 2019; Accepted 19 July 2019
0147-9571/ © 2019 Elsevier Ltd. All rights reserved.

sampling is the first choice. Moreover, clinical samples give reflection of colonization of MAP bacilli in the intestines *etc.* Milk is also good clinical sample to indicate colonization of bacilli and easy to collect. Blood as test sample was the best but is difficult to collect from large ruminants and owners were reluctance for this sample in this part of the world. Target tissues (mesenteric lymph nodes and intestines) were best as test sample for investigating MAP infection and extent of damage by disease processs, in slaughtered and necropsied animals. Typical MAP bacilli were identified based as presence of clumps of short acid-fast bacilli (AFBs). In-view of the high sensitivity, repeatability, capacity to test large number of samples (mass screening) and cost-effectiveness, ELISA is the most widely used assay used to screen herds ('Herd test'), against MAP infection [15]. However, specificity of ELISA test using semi-purified protoplasmic (sPPA) or whole cell lysate of antigens, is compromised [16–18]. MAP infection induces mucosal thickening in small intestines (INT), enlargement of MLNs, serosal lymphatic vessels and mild to marked granulomatous lesions in intestines (INTs) [19]. Infected cows and other animals remain asymptomatic shedders even up to 2–5 years following infection and merely 10–15% of infected animals exhibit clinical symptoms [3,19]. Sub-clinical cases of JD are difficult to diagnose [20,21]. Emerging evidences suggest that in sheep, histo-pathology was the better diagnostic approach to detect sub-clinical infection of MAP [21].

In this study, we evaluated diagnostic efficacy of three screening tests {two antibodies detection tests (newly developed six recombinant secretory proteins based cocktail_ELISA and semi-purified protoplasmic antigens (sPPA) or whole cell lysate based in-house 'indigenous_ELISA kit', and antigen detection tissue microscopy (Acid fast staining) with respect to 'Gold standard' histo-pathology of target tissues (MLN and intestines) for the diagnosis of JD in slaughtered and necropsied goats and slaughtered buffaloes.

2. Material and methods

2.1. Collection of tissue samples

Samples; serum and tissues (lymph nodes and ileum) were collected from goats (n = 77) and buffaloes (n = 40) slaughtered for harvesting of meat during three years (2015–2017) period. Samples were also collected from farm goats (n = 17) died and necropsied to know the cause of death. Samples were driven mainly from the two adjoining Indian states; Uttar Pradesh {Mathura (farm goats were located at Central Institute for Research on Goats), Kosi and Agra} and Rajasthan; Jaipur. Age of animals sampled ranged from1to 6 years. Tissues were collected in 10.0% buffered formalin for histo-pathology. For microscopy, tissues were collected in sterilized poly bags without adding any preservatives.

2.2. Sample processing and tissue microscopy

Tissue samples of INTs and MLNs were collected and preserved in 10% neutral buffered formalin and subjected to histo-pathological examination as per previously described procedure [22]. Tissue sections (4–5 μm) were cut and stained with Haematoxylin and Eosin (H&E) staining [22]. Staining of tissue sections by tissue microscopy (ZN staining) was performed as described previously [23] and part of tissues were kept for molecular assays at 4^0C.

2.3. Histo-pathology (H & E staining)

Histo-pathological lesions were graded as type I, II, III and IV, depending on the type and density of cellular infiltrates (lymphocytes, macrophages and epithelioid cells) in small INTs and MLNs as per Hailat et al. (2010) [24]. 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 grade I with some macrophages and epithelioid cells (more than in grade I) were considered as grade II. Lesions having small amount of lymphocytes with abundant epithelioid cells and macrophages forming micro granulomas 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 granulomas with giant cells were considered as grade IV (Table 2). Tissue sections were considered positive for JD when macrophage infiltrations or epithelioid cells were obvious in the lamina propria of the villi or submucosa.

2.4. Six Recombinant Culture filtrate Proteins (CFP) or secretory proteins (SP)-based cocktail_ELISA

'Indigenous ELISA' (i_ELISA) using semi-Purified Protoplasmic Antigens (sPPA) of MAP obtained from 'S 5' 'Indian bison type' [25] biotype was developed in goats [26] and has been employed in cattle by Sharma et al. (2008) [27] and buffaloes by Yadav et al., (2008) [55]. Six recombinant CFPs; MAP1693c; MAP 2168c; MAP ModD; MAP 85C; MAP Pep AN and MAP Pep AC, based ELISA was used at 6 μg of six r-CFPs (1 μg of each r_CFPs) to develop cocktail ELISA (c_ELISA) [5,28]; and 1 μg of each individual r-CFP to develop recombinant-ELISA (r_ELISA). Serum samples were collected from culture positive (clinically infected) and culture negative animals and used as positive and negative controls, respectively. OD values of the samples were expressed as sample to positive {S/P} ratios [29]. Animals in positive and strong positive categories of the S/P ratios were considered positive for MAP infection or JD. Sensitivity and specificity of c_ELISA was compared with i_ELISA.

S/P ratio and corresponding status of JD:

S/P ratios revealed corresponding status of JD; between 0.00-0.09- Negative (N), 0.1-0.24- Suspected / Borderline (S), 0.25-0.39- Low Positive (LP), 0.4-0.99- Positive (P) and 1.0–10.0- Strong Positive (SP) (Tables 1 and 5) [29].

$$\text{S/Pratio value} = \frac{\text{OD at 450 nm of test serum} - \text{OD at 450 nm of negative control}}{\text{OD at 450 nm of positive control} - \text{OD at 450 nm of negative control}}$$

2.5. Sensitivity and specificity

Sensitivity = True Positive X 100 / True Positive + False Negative
Specificity = True Negative X 100 / True Negative + False Positive

2.6. Statistical analysis

McNemar's test and kappa agreement have applied for the measure the statistical significance between results of two tests (GraphPad software, USA) (Tables 11–13).

3. Results

Two serological (i_ELISA and c_ELISA) tests and two antigen

Table 1
S/P ratios and corresponding status of JD**.

S. No.	S/P Ratio	JD status in animal
1	0.00 – 0.09	Negative
2	0.10 – 0.24	Suspected or Borderline
3	0.25 – 0.39	Low Positive
4	0.4 – 0.99	Positive*
5	1.0 – 10.0	Strong Positive*

* Animals in Positive and Strong Positive categories of S/P ratio were taken as 'Positive'.

** As per Collins (2002).

Table 2
Grading of pathological lesions present in the ileo-caecal junction of intestines and lymph nodes.

Grade	Type of inflammatory cells				
	Lymphocytes	macrophage	Epitheloid cells	PP prol. and crypts replacement	Microgranuloma
I	+ + + +	+ +	+	–	–
II	+ + +	+ + + +	+ + +	Moderate	Yes/no
III	+	+ + +	+ + + +	Severe	Yes
IV (SP)	+	+ + + +	+ + + +	Calcification, caseous necrosis in the LN	Granuloma with giant cells

SP- special, PP Prol.-Peyer's patches proliferation, LN- lymph nodes.

Table 3
Grading of lesions for MAP infection in slaughtered goats, buffaloes and necropsied goats using histo-pathology.

Animals	Tissues	Histo-pathology (Grading of lesions) n (%)				
		Negative	Grade I	Grade II	Grade III	Grade IV
Goats (Slaughtered) n = 77	MLN-77	13 (16.8)	26 (33.7)	23 (29.8)	11 (14.2)	4 (5.1)
	INT-77	16 (20.7)	23 (29.8)	27 (35.0)	8 (10.3)	3 (3.8)
Sub-total A	**154**	**29 (18.8)**	**49 (31.8)**	**50 (32.4)**	**19 (12.3)**	**7 (4.5)**
Buffaloes (Slaughtered) n = 40	MLN-40	11 (27.5)	17 (42.5)	9 (22.5)	2 (5.0)	1 (2.5)
	INT-40	12 (30.0)	16 (40.0)	10 (25.0)	1 (2.5)	1 (2.5)
Sub-total B	**80**	**23 (28.7)**	**33 (41.2)**	**19 (23.7)**	**3 (3.7)**	**2 (2.5)**
Goats (Necropsied) n = 17	MLN-17	3 (17.6)	3 (17.6)	7 (41.1)	3 (17.6)	1 (5.8)
	INT-17	3 (17.6)	5 (29.4)	5 (29.4)	3 (17.6)	1 (5.8)
Sub-total C	**34**	**6 (17.6)**	**8 (23.5)**	**12 (35.2)**	**6 (17.6)**	**2 (5.8)**
Grand Total (A + B + C) n = 134	**268**	**58 (21.6)**	**90 (33.5)**	**81 (30.2)**	**28 (10.4)**	**11 (4.1)**
		58 (21.6)	**210 (78.3)**			

detection (tissue microscopy and tissues histo-pathology) were used to screen samples of 134 animals (77 slaughtered goats, 40 slaughtered buffaloes and 17 necropsied goats). Tissues (134 INTs and 134 MLN) of goats and buffaloes revealed variable grades of lesions of JD in 103 (76.8%) ilea and 107 (79.8%) MLNs (Table 3). 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 clinical cases of JD, especially in goats, ileo-caecal junctions were consistently thickened and corrugated (Figs. 1, 2 and 4). MLNs showed lympho-follicular hyper-plasia causing thickening of cortical area of lymph nodes and enlargement (Figs. 1, 2 and 4). In slaughtered goats and buffaloes and necropsied goats, 33.7, 42.5 and 17.6%, MLNs and 29.8, 40.0 and 29.4%, INTs, respectively had lesions of grade I (Table 3) and were characterized by infiltrations of lymphocytes in larger amount with presence of few macrophages and epithelioid cells without fusion and formation of micro-granuloma (Fig. 9). Whereas, 29.8, 22.5 and 41.1%, MLNs and 35.0, 25.0 and 29.4%m INTs, respectively had lesions of grade II, characterised by infiltration of lymphocytes in relatively lesser amount and higher number of macrophages and epithelioid cells without forming distinct granuloma (Table 3; Fig. 5). While 14.2, 5.0 and 17.6%, MLNs and 10.3, 2.5 and 17.6%, INTs, respectively had lesions of grade III, characterized by severe infiltration of epithelioid cells and macrophages forming distinct microgranulomas with presence of few lymphocytes along with moderate proliferation of Peyer's patches (Table 3; Fig. 6). However, grade IV lesions were found only in 5.1, 2.5 and 5.8%, MLNs and 3.8, 2.5 and 5.8%, INTs, respectively, which had infiltration of lymphocytes, macrophages and abundant epithelioid cells forming sheet like appearance in lamina propria and sub-mucosa of the INT along with severe hypertrophy of Peyer's patches. Multi-nucleated syncytia or giant cells near granuloma were also seen in MLNs (Table 3 and Figs. 7 and 11). Cases were considered as grade IV (special category) having calcification, typical granulomatous structures were observed in the lymph nodes. Cases in grades I and II were of the initial developmental stages, since JD was spectral disease and endemic in herds were kept in suspected category. Whereas, cases in grades III and IV were considered positive for the active disease as per the histo-pathological evaluation.

Tissue samples (INTs and MLNs) from 134 animals (77 slaughtered goats, 40 slaughtered buffaloes and 17 necropsied goats) were screened by tissue microscopy, and 80 (59.7%) tissues from MLNs and 78 (58.2%) from INTs (Table 4, Fig. 3) were positive for acid fast bacilli. In slaughtered goats and buffaloes and necropsied goats, 55.8, 57.5 and 82.3%, MLNs and 54.5, 55.0 and 82.3%, INTs, respectively were positive in tissue microscopy'. While, 27.2, 30.0 and 17.6%, MLNs and 32.4, 27.5 and 29.4%, INTs were in 1+ category; 20.7, 13.7 and 41.1%, MLNs and 19.4, 25.0 and 5 (29.4%), INTs were in 2+ category; 6.4, 0 (0.0) and 17.6%, MLNs and 1.29, 2.5 and 17.6%, INTs were in 3+ category and 1.29, 0.0 and 5.8%, MLNs and 1.29, 0.0 and 5.8%, INTs were in 4+ categories, respectively and were positive for AFB (Table 4). Bacilli were seen either inside the macrophages or outside as extracellular entity.

Table 5, exhibits clinical profile of goats with respect to the status of MAP when OD values were converted to S/P ratio as per Collins (2002) [32]. Cut off for MAP infected animals (Positives) in i_ELISA and c_ELISA, had animals in strong positive (SP) and positive (P) categories.

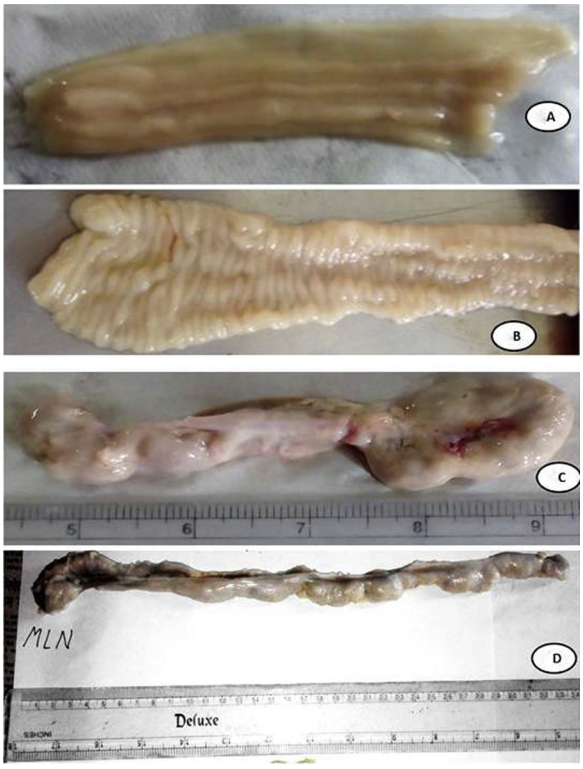

Fig. 1. Gross and lesions present in Ileo-caecal Junction of INTs, MLNs of MAP infected goats: 1A. Generalized thickening and transeversefoldings in Intestine of goat; 1B.Intestine showing corrugation, thickening with folded mucosa in Intestine of goat; 1C.Enlarged, thickened and pulpy MLN in goat; 1D.Enlargement of MLN in goat.

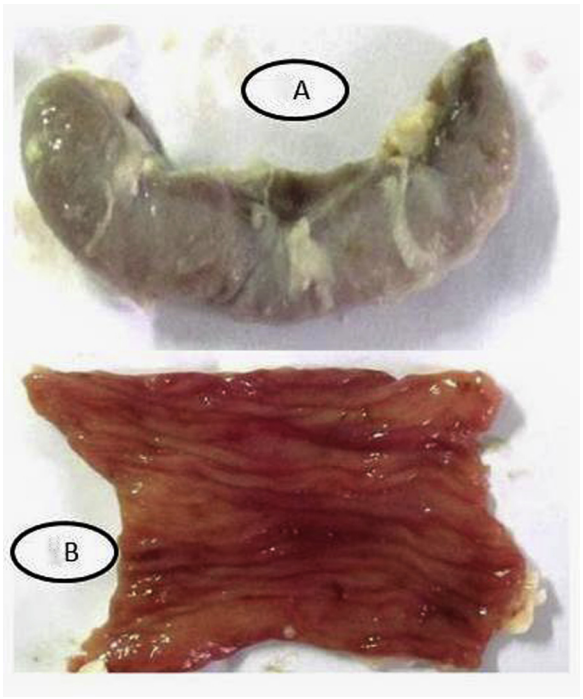

Fig. 2. Gross and lesions present in Ileo-caecal Junction of intestine and mesenteric lymphnode of MAP infected buffalo: 2A. Intestine showing corrugation, generalized thickening and folded mucosa in Intestine of buffalo; 2B.Thickened and pulpy MLN in buffaloes.

Table 4
Tissue microscopy in slaughtered goats and buffaloes and necropsied goats.

Animals	Tissues	Tissue microscopy n (%)				
		Negative	+ 1	+ 2	+ 3	+ 4
Goats (Slaughtered) n = 77	MLN-77	34 (44.1)	21 (27.2)	16 (20.7)	5 (6.4)	1 (1.29)
	INT-77	35 (45.4)	25 (32.4)	15 (19.4)	1 (1.29)	1 (1.29)
Sub-total A	**154**	**69 (44.8)**	**46 (29.8)**	**31 (20.1)**	**6 (3.8)**	**2 (1.29)**
Buffaloes (Slaughtered) n = 40	MLN-40	17 (42.5)	12 (30.0)	11 (13.7)	–	–
	INT-40	18 (45.0)	11 (27.5)	10 (25.0)	1 (2.5)	–
Sub-total B	**80**	**35 (43.7)**	**23 (28.7)**	**21 (26.2)**	**1 (1.2)**	–
Goats (Necropsied) n = 17	MLN-17	3 (17.6)	3 (17.6)	7 (41.1)	3 (17.6)	1 (5.8)
	INT-17	3 (17.6)	5 (29.4)	5 (29.4)	3 (17.6)	1 (5.8)
Sub-total C	**34**	**6 (17.6)**	**8 (23.5)**	**12 (35.2)**	**6 (17.6)**	**2 (5.8)**
Grand Total (A + B + C) n = 134	**268**	**110 (41.0)**	**77 (28.7)**	**64 (23.8)**	13 (4.8)	**4 (1.4)**
		110 (41.0)	**158 (58.9)**			

Low positive (LP) were not taken as positive since MAP infection was endemic in the two livestock species in India. Screening of 117 serum samples (77 goats and 40 buffaloes) revealed 62.3 and 58.9% positive by i_ELISA and c_ELISA, respectively (Table 5). Comparison of c_ELISA and i_ELISA showed that results of positive and negative slaughtered goats and buffaloes were comparable. The 2.5% positive samples of slaughtered goats and buffaloes in i_ELISA were missed by c_ELISA (Table 5).

Of 77 slaughtered goats screened by histo-pathology, tissue microscopy, i_ELISA and c_ELISA, 35.0 and 15.5%, slaughtered goats were positive and negative in all the 4 tests. Highest number of goats (83.1%) were detected in histo-pathology followed by tissue microscopy (63.6%), i_ELISA (40.2%) and c_ELISA (37.6%) (Table 6). Of the 40 slaughtered buffaloes screened by tissue microscopy, histo-pathology, i_ELISA and c_ELISA, 52.5 and 10.0% were positive and negative in all the 4 tests. Highest numbers of buffalows (85.0%) were detected by histo-pathology followed by i_ELISA (65.0%), c_ELISA (60.0%) and tissue microscopy (55.0%) (Table 6). Comparison of results in two test combinations (histo-pathology and tissue microscopy) in slaughtered goats and buffaloes and necropsied goats, 66.2, 57.5 and 70.5% were positive and 15.5, 12.5 and 11.7% were negative, respectively (Table 7). In four test combinations', c_ELISA had lowest sensitivity in slaughtered goats and tissue microscopy had lowest sensitivity in slaughtered buffaloes (Table 6). In the two tests combinations (tissue microscopy and histo-pathology), tissue microscopy had lower sensitivity in slaughtered goats and buffaloes and necropsied goats (Table 7).

3.1. Sensitivity and specificity of tests

A Slaughtered goats

a Comparison of tissue microscopy with histo-pathology: Sensitivity of tissue microscopy and histo-pathology was 79.6 and 98.0% and specificity was 92.3 and 48.0% in slaughtered goats, respectively (Table 8).

b Comparison of tissue microscopy with i_ELISA: Sensitivity of tissue microscopy and i_ELISA was 100.0 and 54.3% and specificity was 59.6 and 100.0% in both the tests in slaughtered goats, respectively (Table 8).

c Comparison of tissue microscopy with c_ELISA: Sensitivity of

Table 5
Evaluation of Indigenous ELISA and Cocktail ELISA using serum of slaughtered goats and buffaloes.

Animals (n)	i_ELISA, n (%)					c_ELISA, n (%)				
	Negative			Positive		Negative			Positive	
	Negative (N)	Suspected (S)	Low Positive (LP)	Positive (P)	Strong Positive (SP)	Negative (N)	Suspected (S)	Low Positive (LP)	Positive (P)	Strong Positive (SP)
Goats (77)	–	17 (22.0)	15 (19.4)	30 (38.9)	15 (19.4)	11 (14.2)	16 (20.7)	7 (9.0)	33 (42.8)	10 (12.9)
Buffaloes (40)	1 (2.5)	9 (22.5)	2 (5.0)	26 (62.5)	2 (5.0)	9 (22.5)	2 (5.0)	3 (7.5)	16 (40.0)	10 (25.0)
Sub-total A (117)	**1 (0.8) 44 (37.6)**	**26 (22.2)**	**17 (14.5)**	**56 (47.8) 73 (62.3)**	**17 (14.5)**	**20 (17.0) 48 (41.0)**	**18 (15.3)**	**10 (8.5)**	**49 (33.3) 69 (58.9)**	**20 (17.0)**

i_ELISA- Indigenous ELISA, c_ELISA- Cocktail ELISA, N- Negative, S- Suspected, LP- Low Positive, P- Positive, SP- Strong Positive.

tissue microscopy and c_ELISA was 100.0 and 52.0% and specificity was 55.7 and 100.0%, respectively (Table 8).

d Comparison of histo-pathology with i_ELISA: Sensitivity of histo-pathology staining and i_ELISA was 100.0 and 46.8% and specificity was 27.6 and 100.0%, respectively (Table 8).

e Comparison of histo-pathology with c_ELISA: Sensitivity of histo-pathology and c_ELISA was 100.0 and 45.3% and specificity was 27.8 and 100.0%, respectively (Table 8).

f Comparison of i_ELISA with c_ELISA: Sensitivity of i_ELISA and c_ELISA was 93.1 and 87.1 and specificity was 91.6 and 95.6% in both the test (Table 8).

B Slaughtered Buffaloes

a Comparison of tissue microscopy with histo-pathology: Sensitivity of tissue microscopy was 67.6 and 95.8% and specificity was 83.3 and 31.2% in slaughtered buffaloes, respectively (Table 9).

b Comparison of tissue microscopy with i_ELISA: Sensitivity of tissue microscopy and i_ELISA was 82.1 and 100.0% and specificity was 100.0 and 70.5%, respectively (Table 9).

c Comparison of tissue microscopy with c_ELISA: Sensitivity of tissue microscopy and i_ELISA was 96.4 and 95.8% and specificity was 41.6 and 83.3%, respectively (Table 9).

d Comparison of histo-pathology with i_ELISA: Sensitivity of histo-pathology and i_ELISA was 96.4 and 79.4% and specificity was 41.6 and 83.3%, respectively (Table 9).

e Comparison of histo-pathology with c_ELISA: Sensitivity of histo-pathology and i_ELISA was100.0 and 76.4% and specificity was 42.8 and 100.0%, respectively (Table 9).

f Comparison of i_ELISA with c_ELISA: Sensitivity of i_ELISA and c_ELISA was 100.0 and 92.8% and specificity was 85.7 and 100.0%, respectively (Table 9).

C Necropsied goats

a Comparison of tissue microscopy with histo-pathology: Sensitivity of tissue microscopy and histo-pathology staining was 85.7 and 92.3% and specificity was 66.6 and 50.0% in necropsied goats, respectively (Table 10).

3.2. Statistical analysis

Statistical analysis was conducted using Mc Nemar test. P Value and kappa agreement were calculated for various field samples vis-à-vis combination of diagnostic techniques compared during the current study.

a **Slaughtered goats:** P values were, 0.0033, 0.0001, 0.0001, 0.0001, 0.0001 and 0.6831 and Kappa agreements, 0.526, 0.489, 0.450, 0.230, 0.219 and 0.836 in tissue microscopy - histo-pathology; tissue microscopy - i_ELISA; tissue microscopy - c_ELISA; histo-pathology - i_ELISA, histo-pathology - c_ELISA and i_ELISA - c_ELISA, respectively. Strength of agreement was perfect, poor, poor, poor, poor and perfect, respectively (Table 11).

b **Slaughtered buffaloes:** P values were, 0.0094, 0.0736, 0.6171, 0.0771, 0.0133 and 0.4795 and Kappa agreements, 0.302, 0.734, 0.787,0.444, 0.494 and 0.886 in tissue microscopy - histo-pathology staining; tissue microscopy - i_ELISA; tissue microscopy - c_ELISA; histo-pathology - i_ELISA, histo-pathology - c_ELISA and i_ELISA - c_ELISA, respectively. Strength of agreement was fair, substantial, substantial, moderate, moderate and perfect, respectively (Table 12).

c **Necropsied goats:** P values were 1.0000 and Kappa agreements, 0.463 in tissue microscopy - histo-pathology. Strength of agreement was moderate (Table 13).

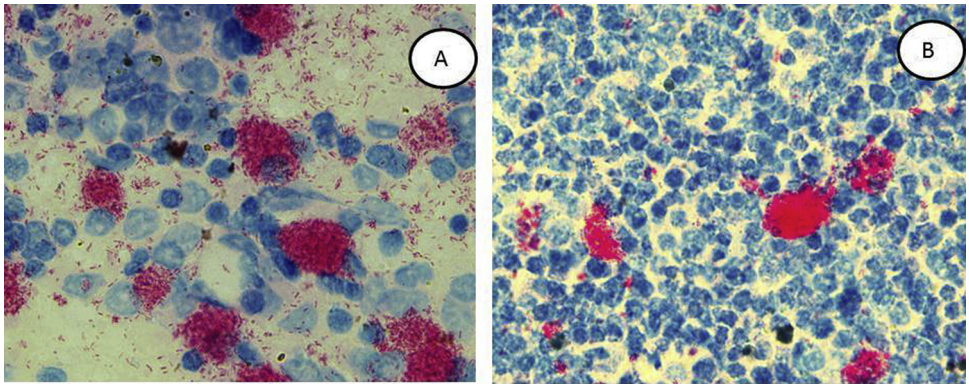

Fig. 3. Scrapping smears: (3A) Ileo-caecal Junction of intestine and (3B) mesenteric lymphnode showing typical MAP bacilli by Ziehl-Neelsen stain 100 × .

Table 6
Comparison of tissue microscopy, histo-pathology, i_ELISA and c_ELISA in slaughtered goats and buffaloes.

Tests (n)	Combinations, n (%)									
	1	2	3	4	5	6	7	8	9	10
Tissue microscopy	+	–	+	–	–	+	–	+	–	+
Histo-pathology	+	–	–	+	–	+	+	+	+	+
i_ELISA	+	–	–	–	+	–	+	–	+	+
c_ELISA	+	–	–	–	–	–	–	+	+	–
Slaughtered goats	**27**	**12**	**1**	**13**	**0**	**18**	**0**	**2**	**0**	**4**
(n = 77)	**(35.0)**	**(15.5)**	**(1.2)**	**(16.8)**	**(0.0)**	**(23.3)**	**(0.0)**	**(2.5)**	**(0.0)**	**(5.1)**
Slaughtered buffaloes	**23**	**4**	**1**	**7**	**1**	**0**	**1**	**0**	**3**	**0**
(n = 40)	**(57.5)**	**(10.0)**	**(2.5)**	**(17.5)**	**(2.5)**	**(0.0)**	**(2.5)**	**(0.0)**	**(7.5)**	**(0.0)**

Table 7
Comparison results of tissue microscopy, histo-pathology in slaughtered goats & buffaloes and necropsied goats.

Tests	Combinations, n (%)			
	1	2	3	4
Tissue microscopy	+	–	+	–
Histo-pathology	+	–	–	+
Slaughtered goats	**51**	**12**	**1**	**13**
(n = 77)	**(66.2)**	**(15.5)**	**(1.2)**	**(16.8)**
Slaughtered buffaloes	**23**	**5**	**1**	**11**
(n = 40)	**(57.5)**	**(12.5)**	**(2.5)**	**(27.5)**
Necropsied goats	**12**	**2**	**1**	**2**
(n = 17)	**(70.5)**	**(11.7)**	**(5.8)**	**(11.7)**

Table 8
Comparative sensitivity and specificity of tests in slaughtered goats.

S.No.	Test	TP	TN	FP	FN	Sensitivity (%) & Specificity (%)
1	Tissue microscopy	51	12	1	13	79.6 & 92.3 %
	Histo-pathology	51	12	13	1	98.0 & 48.0 %
2	Tissue microscopy	31	25	21	0	100.0 & 54.3 %
	i_ELISA	31	25	0	21	59.6 & 100.0%
3	Tissue microscopy	29	25	23	0	100.0 & 52.0 %
	c_ELISA	29	25	0	23	55.7 & 100.0%
4	Histo-pathology	30	13	34	0	100.0 & 27.6%
	i_ELISA	30	13	0	34	46.8 & 100.0%
5	Histo-pathology	29	13	35	0	100.0 & 27.8%
	c_ELISA	29	13	0	35	45.3 & 100.0%
6	i_ELISA	27	44	4	2	93.1 & 91.6%
	c_ELISA	27	44	2	4	87.1 & 95.6%

Table 9
Comparative sensitivity and specificity of tests in slaughtered buffaloes.

S. No.	Test	TP	TN	FP	FN	Sensitivity (%) & Specificity (%)
1	Tissue microscopy	23	5	1	11	67.6 & 83.3%
	Histo-pathology	23	5	11	1	95.8 & 31.2%
2	Tissue microscopy	23	12	0	5	82.1 & 100.0%
	i-ELISA	23	12	5	0	100.0 & 70.5%
3	Tissue microscopy	23	13	1	3	88.4 & 92.8%
	c-ELISA	23	13	3	1	95.8 & 81.2%
4	Histo-pathology	27	5	7	1	96.4 &41.6%
	i-ELISA	27	5	1	7	79.4 &83.3%
5	Histo-pathology	26	6	8	0	100.0 & 42.8%
	c-ELISA	26	6	0	8	76.4 & 100.0%
6	i-ELISA	26	12	2	0	100.0 & 85.7%
	c-ELISA	26	12	0	2	92.8 &100.0%

Table 10
Comparative sensitivity and specificity of tests in necropsied goats.

S. No.	Test	TP	TN	FP	FN	Sensitivity (%) & Specificity (%)
1	Tissue microscopy	12	2	1	2	85.7 & 66.6%
	Histo-pathology	12	2	2	1	92.3 & 50.0%

4. Discussion

Johne's disease being insidious, chronic and stress dependant disease had spectral profile. Besides virulence of pathogen, development of clinical disease was dependent on many factors like stress for the progression. Animals get MAP infection during pregnancy and at birth through feeding of colostrum and milk, however, clinical symptoms were non-specific (weight loss and diarrhoea), and appeared much later (2–5 years or more). Meanwhile disease slowly progresses inside tissues, and animals develop weakness and diarrhoea at much later stage. Whereas, human beings exhibit symptoms of weakness, low grade fever, frequent bowel movement, weight loss, *etc.*). Diagnosis of spectral diseases like JD, is very challenging, since in a herd at particular time animals are in different stages of infection. Therefore, no single test can identify all the infected animals at any given time. Therefore, at least two tests (one screening and one confirmatory), are needed for the diagnosis of JD. Depending on our requirements and purpose, we can select any one or more than one test combination, from the variety of tests available and standardized at our laboratory. In view of the long incubation period, intermittent shedding of bacilli, poor body condition (weakness and weight loss), antibody response is weak and variable. Poor nutrition and stressful conditions (over-crowding, production stress, high incidence of other infectious / diseases, especially in developing countries like India), JD has become highly endemic in herds and flocks of domestic livestock. Problems with existing global commercial tests / kits is their low sensitivity [30,31] and high cost. Therefore, depending on the MAP bio-types, epidemiology of disease and intensity of MAP infection, pathogenicity, *etc.*, every country should endeavour to develop own tests using locally prevalent biotypes. In India, using our native predominant biotype (Indian Bison type), we have developed and standardized variety of diagnostic tests for detection of antigen / bacilli (tissue microscopy, culture, i_FAT, Immuno-histochemistry based tests, Nano-Immuno Rapid test, PCR, PCR_RE, LAMP_PCR, TaqMan probe q_PCR, SYBR Green Real Time PCR) and antibodies (i_ELISA, Latex Agglutination Test, dot_ELISA *etc.*). Therefore, we continue to validate our tests with 'gold standard test' like culture or histo-pathology. In the present study we compared newly developed c_ELISA using six recombinant secretory proteins, i_ELISA and tissue microscopy with histo-pathology of the target tissues (INTs and MLNs) in goats and buffaloes. Despite heavy to very heavy slaughter rate of goats and buffaloes (in order to meet the requirement of meat both for internal and external markets), JD continues to be endemic in two livestock species [4].

MAP Infection leads to extensive damages in the infected tissues

Table 11
Comparison between tests in slaughtered goats using Mc-Nemar test and Kappa agreement.

Sn	Tests compared	P value		Kappa value	Strength of agreement	95% Confidence interval
		Status	Value			
1	Tissue microscopy *vs* Histo-pathology	Very significantly different	0.0033	0.526	Moderate	0.323 to 0.729
2	Tissue microscopy *vs* i-ELISA	Extremely significantly different	0.0001	0.489	Moderate	0.329 to 0.650
3	Tissue microscopy *vs* c-ELISA	Extremely significantly different	0.0001	0.450	Moderate	0.293 to 0.607
4	Histo-pathology *vs* i-ELISA	Extremely significantly different	0.0001	0.230	Fair	0.106 to 0.353
5	Histo-pathology *vs* c_ELISA	Extremely significantly different	0.0001	0.219	Fair	0.099 to 0.338
6	i-ELISA *vs* c_ELISA	Not significantly different	0.6831	0.836	Perfect	0.711 to 0.962

Kappa value (0.0 – 0.20, poor; 0.21- 0.40, fair; 0.41 -0.60, moderate; 0.61 - 0.80, substantial and 0.81–100, perfect).

(INTs and MLN) much before the development of clinical signs, therefore, histo-pathology isconsidered 'Gold standard' for detection of MAP infection or diagnosis of JD. We estimated sensitivity and specificity of newly developed six recombinant secretary proteins based ELISA (c_ELISA) and robust indigenous ELISA (i_ELISA) kit as antibody detection tests and tissues microscopy (antigen detection test).

On necropsy most significant gross findings were mild to moderate, focal / diffuse thickening of terminal ileum especially at ileo-caecal junction with or without transverse / longitudinal corrugations. MLNs were severely enlarged approximately double in the size and were found to be oedematous and juicy. In this study, 76.8% (101/134) INTs and 80.5% (108/134) MLN showed various grades of histo-pathological lesions which were characterized on the basis of infiltration of mononuclear cells consisting of lymphocytes, macrophages and epithelioid cells in lamina propria and sub-mucosa of INT and cortex and medulla of MLNs with formation of micro-granuloma. Affected part of the INTs revealed degeneration and partial to complete denudation of lining of epithelial cells forming naked villi in this study and similar findings have been reported by earlier workers [32,33]. **V**illi exhibited variable changes that included dilated lacteals, villous distortion including thickening and fusion of villi were observed [34]. **S**imilar lesions such as thickening of the intestinal villi with flat and wide tips, infiltration of lymphocytes, macrophages and epithelioid cells in lamina propria of INTs were also observed in this study. MLNs showed lympho-follicular hyperplasia causing thickening of cortical area of lymph nodes with variable degree of infiltration of lymphocytes, macrophages and epitheloid cells with or without formation of micro-granuloma (Figs. 4–10).

Table 13
Comparison between tests in necropsied goats using Mc-Nemar test and Kappa agreement.

Sn	Tests compared	P value		Kappa value	Strength of agreement	95% Confidence interval
		Status	Value			
1	Tissue microscopy *vs* Histo-pathology	Not significantly different	1.0000	0.463	Moderate	−0.049 to 0.975

Kappa value (0.0–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial and 0.81–100, perfect).

Grade I lesions were found in 32.8% (44/134) INTs and 34.3% (46/134) MLNs (Table 3), characterized by infiltration of large number of lymphocytes with few epithelioid cells in mucosa / sub-mucosa of INTs and these lesions represented early stages of the infection and persistence of MAP resulted in continuous antigenic stimulation and recruitment of inflammatory cells [35]. Grade II lesions were found in 42 (31.3%) INTs and 39 (29.1%) MLNs 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 occurrence of antagonistic pathological processes and macrophage infiltration was much correlated with the presence of acid-fast bacteria [32,33]. Moreover, 12 (8.9%) cases

Table 12
Comparison between tests in slaughtered buffaloes using Mc-Nemar test and Kappa agreement.

Sn	Tests compared	P value		Kappa value	Strength of agreement	95% Confidence interval
		Status	Value			
1	Tissue microscopy *vs* Histo-pathology	Very significantly different	0.0094	0.302	Fair	0.040 to 0.565
2	Tissue microscopy *vs* iELISA	Not quite significantly different	0.0736	0.734	Substantial	0.524 to 0.944
3	Tissue microscopy *vs* c-ELISA	Not significantly different	0.6171	0.787	Substantial	0.591 to 0.984
4	Histo-pathology *vs* i-ELISA	Not quite significantly different	0.0771	0.444	Moderate	0.141 to 0.748
5	Histo-pathology *vs* c_ELISA	Significantly different	0.0133	0.494	Moderate	0.223 to 0.764
6	i-ELISA *vs* c-ELISA	Not significantly different	0.4795	0.886	Perfect	0.734 to 1.000

Kappa value (0.0 – 0.20, poor; 0.21- 0.40, fair; 0.41 - 0.60, moderate; 0.61 - 0.80, substantial and 0.81–100, perfect).

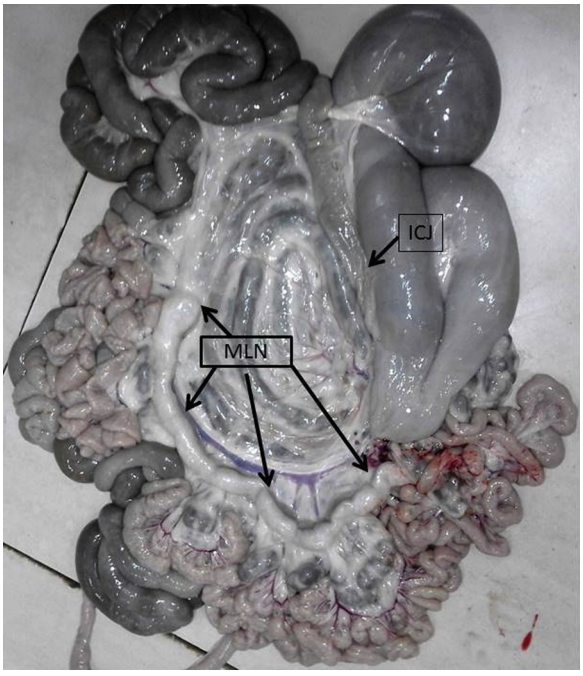

Fig. 4. Gross Photographs of goat mesenteric area: swollen ileo-caecal junction of intestines and enlarged and swollen mesenteric lymph node.

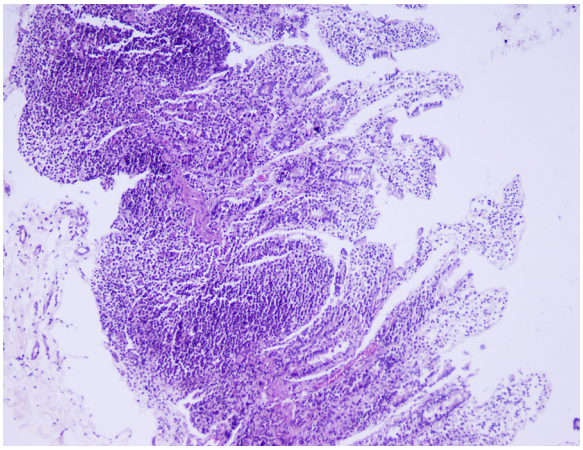

Fig. 5. Goat intestine showing severe infiltration of mononuclear cells without microgranuloma formation (Grade III lesions). histo-pathology 100 × .

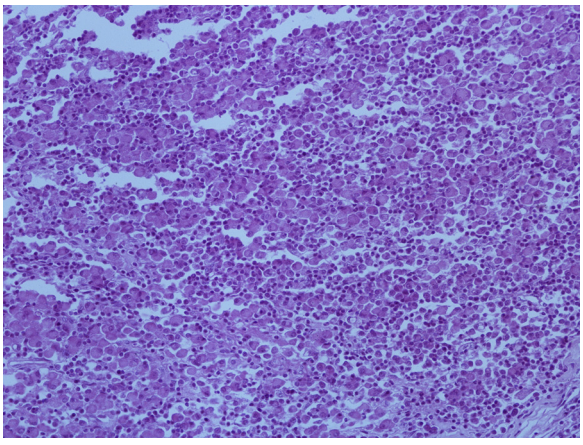

Fig. 6. Intestine of goat showing severe infiltration of epithelioid cells with few giant cells formation (Grade IV lesions), histo-pathology 200 × .

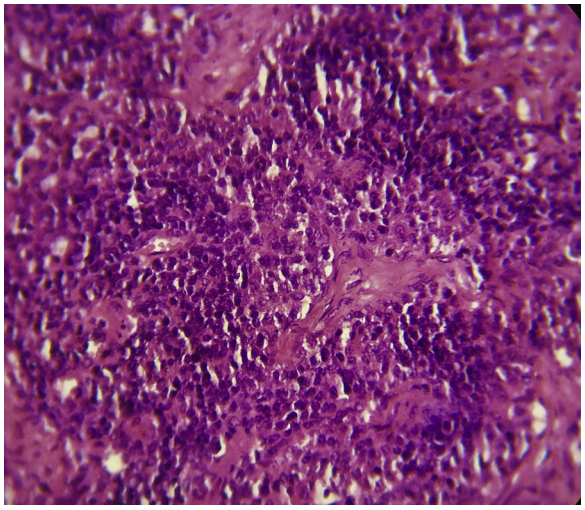

Fig. 7. Mesenteric Lymph Node showing severe infiltration of macrophages with vesicular nucleus (Grade IV lesions) in buffalo histo-pathology 400 × .

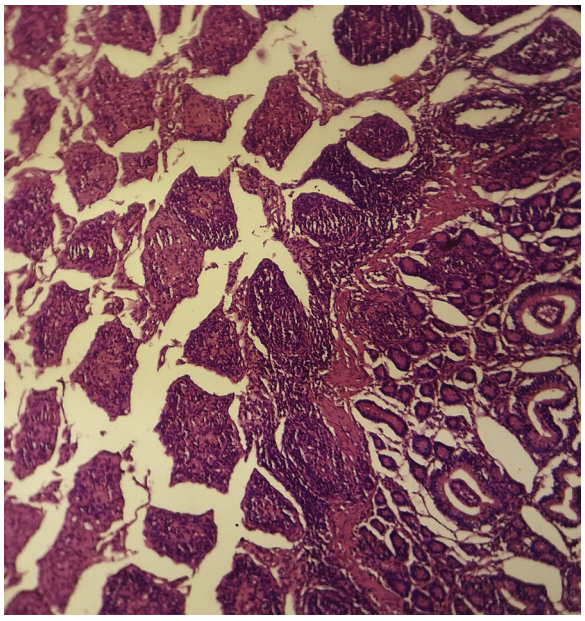

Fig. 8. Intestine showing infiltration of macrophages with degeneration of crypts filled with oedema fluid (Grade III lesions) in buffalo histo-pathology 400 × .

from INTs and 16 (11.9%) from MLNs showed grade III histological lesions, while, only 5 (3.7%) cases from INTs and 6 (4.4%) from MLNs showed grade IV histo-pathological lesions. Grade III and IV histological lesions were characterized by severe infiltration of epithelioid cells with formation of distinct micro-granuloma, while 'giant cells' were also observed in grade IV lesions (Table 3). Lesions found in the current study were earlier described in multi-bacillary cases of ovine paratuberculosis [21,36,37], Gross lesions were clearly visible in lesions of grade III and IV. However, another study reported, gross lesions were clearly visible only in sheep with type 3b and 3c lesions [38].

Accumulation of macrophages and epithelioid cells and formation of granuloma in cortex and medulla suggested that mycobacteria may have entered lymph nodes through afferent lymphatic with spread of infection subsequently to para-cortex through sub-capsular sinuses and then migrated to medullary areas in later stage of infection. Distinct granuloma at places were formed and follicles became depleted after chronic outflow of lymphocytes in the lymph node conform the findings

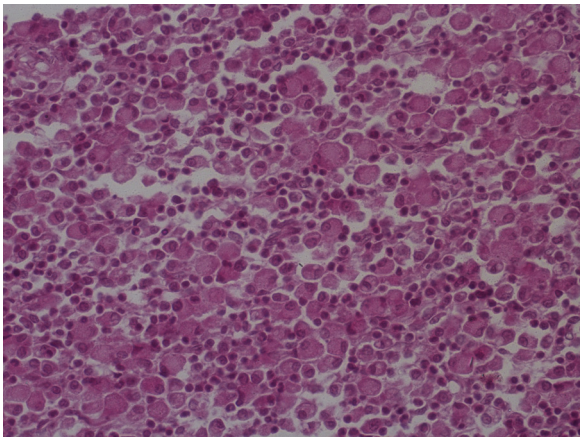

Fig. 9. Severe infiltration of epithelioid cells with few giant cells formation (Grade IV lesions) in intestine of goat. histo-pathology 400x.

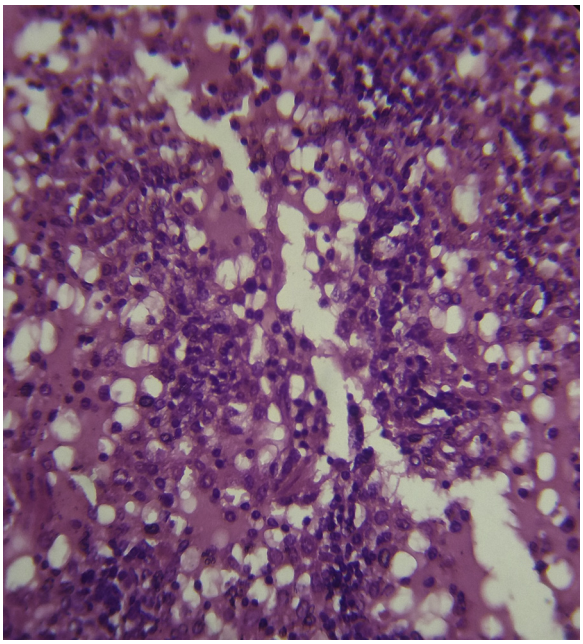

Fig. 10. Goat MLN showing infiltration of vesicular macrophages with oedema fluid (Grade III lesions). histo-pathology 400x.

of several workers who reported similar development of lesions in lymphoid organs in paratuberculosis in sheep and goats [32,38]. In the present study mucosal thickening and corrugations were attributed to mononuclear cell infiltration and similar findings were previously reported which deciphered that inflammatory cell infiltrates were responsible for much of the increased thickness in the mucosa and submucosa combined with villous atrophy [36,39].

Four paired specimens of INTs and MLNs from slaughtered goats with immune-histo-chemical tests on paraffin-embedded tissue sections, using MAP hyper-immune serum. Immuno-histochemical techniques detected positive samples and produced more clearly visible reactions than did acid-fast staining.

In present study, a total of 158 (58.9%) histo-pathological tissues revealed the presence of acid-fast bacilli [81 (60.4%) INTs and 77 (57.4%) MLNs] (Table 4). In accordance with our findings, Clarke et al. (1996) reported 50.0% pauci-bacillary cases positive in tissue microscopy [36]. In this study, 77/268 (28.7%), 64/268 (23.8%), 13/268 (4.8%) and 4/268 (1.4%) tissues found AFB positive were from grade I, II, III and IV lesions, respectively (Fig. 3).

ELISA is efficient in detecting smallest amounts of antibodies

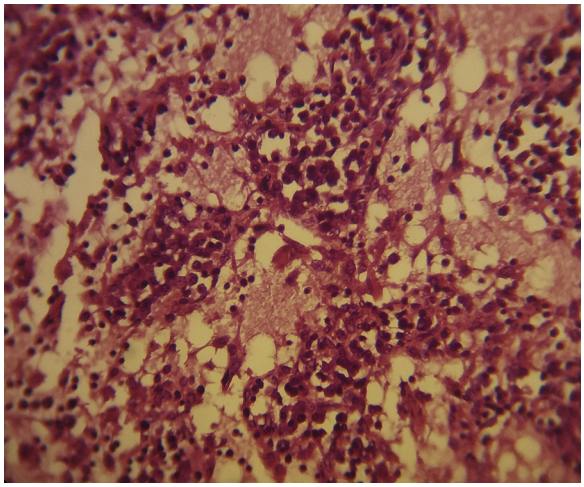

Fig. 11. Goat MLN showing loose aggregates of mixed population of macrophages, epitheliod cells with some cells fuse to form giant cells (Grade III lesions). histo-pathology 400x.

therefore has highest sensitivity among all the serological tests used for MAP [40]. Removal of cross-reacting antibodies by absorbing sera with *Mycobacterium phlei* has improved the specificity of ELISA [41]. However, over the years in India, there is change in the microflora of GIT of domestic livestock, due to the intensification of livestock husbandry from extensive system and disappearance of forests and access to forest area. Some studies reported 98.8 [42], 99.7 [43] and 97.4% [44] specificity and sensitivity of the assay was highly compromised because of foreign nature of the strain of MAP used as antigen source [45]. In general, sensitivity of the ELISA test has been found to be highest in animals with clinical signs of the disease, since antibody titers were higher in the clinical stages of the infection [46,47]. Major benefit of these tests is easy mass scale screening of animals. Selection of antigens is the major challenge in development of pathogen specific ELISA test and sensitive detection. Antibodies produced against shared epitopes of closely related species of organism can contribute to cross-reactivity and exhibit false-positive results and fluctuations in antibody titers and hence development of ELISAs with optimum specificity for the entire mycobacterial diseases, together with human tuberculosis (*Mycobacterium tuberculosis*), leprosy (*Mycobacterium leprae*), bovine tuberculosis (*Mycobacterium bovis*), as well as JD (MAP) is challenging. At the Central Institute for Research on Goats (CIRG), Mathura, India, an ELISA was initially standardized by harvesting antigen from an advance case of clinical JD in a goat followed by using ‘Tepes strain’ of MAP used for making Johnin at IVRI, Izatnagar, India and subsequently used own culture-based antigens [4,48] from 'S 5' strain of MAP, a new biotype, recorded first time in the world [25]. Based on our experience of working on JD since Dec., 1984, MAP bacilli takes long time in doubling (multiplication), use of semi-purified whole cell protoplasmic antigen from locally prevalent immuno-dominant biotype (Indian Bison type) has been found to be best both as screening test and for the diagnosis of MAP infection in domestic livestock [4]. This 'indigenous ELISA test' has since been successfully employed for screening of serum and milk samples of all the domestic livestock species and human beings for the detection of MAP infection and also for the monitoring of the response to the 'indigenous vaccine' developed using this strain (S 5) of goat origin [54]. Based on the likely hood ratio method of Collins (2002) has helped to convert this assay into an ‘Indigenous ELISA kit’ [29]. Ideal characteristics of 'diagnostic antigens' should be the high antigenic property, uniqueness to MAP, easy recognition of infected animals in all the stages of the disease (early/ late stages, sub-clinical/ clinical stages) by remaining recognizable throughout the course of the infection. Diagnostic test for MAP using only immuno-dominant antigens, should use a cocktail of antigens in view of the spectral nature

[49], long range of heterogeneous MHC molecules present in various out-bred animals [49] and differentiate range of antigens expressed by MAP [50]. Mon et al. (2014) recommended use of individual antigens that were able to identify a subset of clinically infected animals. however, cocktail of antigens could be a superior candidate for serological diagnosis of JD [51]. In this study, screening sera samples of 77 goats and 40 buffaloes revealed, 73 (62.3%) and 69 (58.9%) were positive for MAP infection by i_ELISA and c_ELISA, respectively. Comparison of c_ELISA with i_ELISA showed that results of positive and negative slaughtered goats and buffaloes were comparable. But 2 (2.5%) samples positive in i_ELISA, were missed by c_ELISA in goats serum. Similar results were observed in slaughtered buffaloes (Table 5).

Using semi-purified protoplasmic antigen (sPPA) of goat origin harvested from 'Indian Bison type' biotype of MAP (strain 'S 5') based 'i_ELISA' has been standardized for four domestic livestock species [4]. Large number of studies exist comparing sensitivity and specificity of ELISA assays (available commercially or with different scientists) for the screening and diagnosis of MAP infection in cattle and sheep [31,37,45,52]. However studies are limited with respect to goats and buffaloes, since both being Asian animals. i_ELISA had significantly higher sensitivity as compared to commercially available ELISA kits [30,31,45]. I_ELISA also correlated well with culture and was good screening test for the domestic livestock [26,27]. In another study, sensitivity and specificity of i_ELISA was 66.6 and 75.0% and 68.1 and 66.6% with respect to tissue culture and IS900 PCR; respectively [53]. Kumar et al., (2006), examined sensitivity of fecal microscopy with fecal culture and serum i_ELISA and was 90.0 and 65.0%, respectively and agreement was substantial with both tests [56]. Other studies also reported that i_ELISA kit had improved detection rate of MAP in test samples using multiple tests. Using i_ELISA, the detection rate of MAP was increased considerably. Using i_ELISA, the bio-load of MAP recorded increase from 28.9 to 47.0% (Singh et al., 2014) [4]. Yadav et al. (2008) used i_ELISA for the screening of slaughtered buffaloes abd none and 46.7% animals were positive using purified protoplasmic antigen (PPA) of bovine origin (Allied Monitor Inc., USA) and sPPA of native strain (S5) biotype as 'Indian Bison type' biotype of MAP, respectively [55]. This study showed buffaloes being Asian animal exhibited very low sensitivity with commercial antigen based on foreign (US 'cattle type biotype') strain (Allied Monitor Inc., USA). Kumar et al. (2006) indicated greater sensitivity of species specific antigens [62]. Chaubey et al. (2015) reported i_ELISA (g-ELISA) with respect to commercial antigen based b_ELISA and commercial sr_ELISA had 100.0 and 100.0% sensitivity and 44.4% and 11.4% specificity, respectively [45]. Pahangchopi et al. (2014) reported superiority of the antigens used in i_ELISA harvested from MAP strain (S 5), in AGPT as compared commercial antigen (ATCC 19698, UK used for making Johnin in India [52]. Chaubey et al. (2018) compared sensitivity and specificity of EV ELISA with i_ELISA, was less sensitive [57]. These studies highlighted lowered sensitivity as major problem of PPA antigens from foreign biotypes and used in commercial ELISA kits. Antigen source and purification were major factors crucial for sensitivity and specificity in ELISA kits. Secreted proteins have been acknowledged to play central roles in bacterial-host interactions. Secreted proteins present in culture filtrate of MAP, have been focus of this study since considered to be immune-dominant and involved in inducing protective immunity [5]. MAP specific secretary antigens can serve as markers for early diagnosis of JD. Comparing secretary proteins with other cellular proteins showed greater sero-reactivity in MAP infected animals. Secretary proteins are also focus in DIVA based diagnostics and vaccine development. Range of new immunologically important secretary antigens have been identified in MAP using 2D-gel electrophoresis, chromatography, mass spectrometry and peptide mass finger printing. Secretary antigens have major impact on development of novel sero-diagnostic techniques with improved sensitivity and specificity [58–61]. Cho et al. (2007) examined serum from infected cattle for immune-blotting which showed infected serum samples reacted strongly with secretary antigens as compared to antigens of intracellular origin [62]. Sensitivity of sero-diagnostic test improved with use of MAP culture filtrate (CF)/ secretory proteins and similarly in case of other mycobacterial pathogens, including *M. bovis* and *M. tuberculosis* [63–65]. Use of MAP CF antigens in ELISAs increased assay sensitivity by 25% over commercial ELISA's for low MAP shedding animals [66]. Recently, early diagnosis was addressed using experimentally infected calves and screening for antibody responses to a panel of 96 recombinant MAP antigens [67]. Antibody responses were detected as early as 70 days post-infection. However, fluctuations in antibody responses and epitope specificity were observed over 321days [68]. The variability in achieving peak titer varied as per the damage to the lymphoid system (MLN, Peyer's patches) in the infected animals [69]. In Indian conditions, animals in all the four livestock species are endemic for MAP infection [4], therefore normal condition of animals (physical and internal, especially lymphoid tissues) is weak and very poor, which leads to slower response to MAP infection. Many times animals escape the cut off S/P ratio due to poor antibody response. Therefore in Indian conditions, where MAP infection is endemic and condition of domestic livestock is poor due to low grade / quality and in-sufficient nutrition, production and environmental stress *etc.*, the cut-off for positive S/P ratio in ELISA assays (i_ELISA and c_ELISA) should be dynamic and decided herd to herd and animal to animal. However, it may not be practical, therefore current focus is to first get rid of super-shedders or animals in high cut-off range (Positives and strong positives in S/P ratio) and in second and third stages these cut-offs can be lowered to include low positives as positives. If we include at this stage, the number of animals positive will be too high to manage. Some studies suggested that secreted proteins may be better as solid-phase ELISA antigens resulting in a more sensitive assay [62,69]. Pradenas et al. reported that most CF proteins have low molecular weight and reacted strongly with sera from culture-positive cases of JD [70]. They observed a high degree of difference in CF protein immune reactivity in MAP infected animals. Serum samples from cattle with clinical JD or heavy fecal shedders of bacilli reacted more intensively to CF proteins. Infected goats and sheep serum samples showed reactivity with CF proteins tested in immune-blots. These results suggested that a cocktail of CF proteins of MAP could be good candidates as antigens for diagnosis of JD. Dheenadhayalan et al. (2002) also reported immunogenicity of five recombinant antigens, namely MAP 2411,ClpP (MAP 2281c), Ppa (MAP 0435c), 990 MAP0593c and GreA(MAP 1027c) [71]. These recombinant antigens were tested with serum samples from 41 sheep with known MAP infection and 41 non infected control sheep. Two of these antigens, MAP 0593 c and ClpP, reacted against 58.5% and 46.3% test positive sera and 12.1% and 4.9% of the 995 negative control sera, respectively. An earlier study tested recombinant antigens of Ag85A, Ag85B, Ag85C and SOD in ELISA with sera from 60 MAP shedding cows and 22 non-shedding cows [61]. Antigens of the Ag85 complex and SOD showed high reactivity against sera from the MAP shedding cows and little reactivity against sera from the non-shedding cows. Otherwise also commercial production of ELISA kits using recombinant proteins will have more uniform results as compared to ELISA kits developed from MAP strain in continuous passage (Strain S 5 of MAP, Indian Bison Type) and may not show uniform results and may vary from batch to batch, which is not desirable in commercial kits.

5. Conclusion

Study concluded that the sensitivity of histo-pathology was highest as compared to other routinely used tests for the diagnosis of MAP infection. Specificity of newly developed c_ELISA test was higher as compared to i_ELISA and exhibited better profiling of results using this newly developed assay (c_ELISA). Histo-pathology, though highly sensitive could be employed to finally assess the status of disease based on the pathological lesions and helped in determined the limits of detection of i_ELISA and c_ELISA, which was comparable. c_ELISA had better

specificity as compared to i_ELISA, using six recombinant secretory antigens.

Ethical approval

Central Institute for Research on Goats, Makhdoom, Mathura ethical committee chaired by Member Secretary, Institutional Animal Ethics committee (IAEC) and The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi has approved works under reference number IAEC/CIRG/16-17 dated12.05.2016 and confirmed that this project do not have any ethical issue. Serum, tissues samples from animals were collected/ received only for laboratory analysis. We have avoided unnecessary pain and suffering of the animals. Samples were not collected from endangered or protected species.

Declaration of Competing Interest

No conflict of interest to declare.

Acknowledgements

Authors are thankful to the Director, CIRG, Makhdoom, Mathura for providing necessary facilities for performing the research and Dr. Aarti Marskole, Mr. Sahzad and Dr. Mansi Srivastava for their assistance in this work. I am also thankful to Indian Council of Medical Research (ICMR), New Delhi, India for providing funds under grant number 5/8/5/28/TF/2013/ECD-I for this work.

References

[1] A. Tiwari, J.A. Van Leeuwen, S.L. McKenna, G.P. Keefe, H.W. Barkema, Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds, Can. Vet. J. 47 (2006) 874–882.

[2] S.V. Singh, S. Gupta, K.K. Chaubey, K.D. Rawat, N. Kumar, J.S. Sohal, S. Singh, R. Tiwari, S. Chakraborty, K. Dhama, Johne's disease (JD) in a high yielding Holstein friesian cattle dairy farm in India, J. Biol. Sci. 14 (2014) 195–203.

[3] R. Garg, P.K. Patil, S.V. Singh, S. Sharma, R.K. Gandham, A.V. Singh, G. Filia, P.K. Singh, S. Jayaraman, S. Gupta, K.K. Chaubey, R. Tiwari, M. Saminathan, K. Dhama, J.S. Sohal, Comparative evaluation of different test combinations for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis*. Infecting dairy herds in India, Bio. Med. Res. Intern. 2015 (2015) 1.

[4] S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal, N. Kumar, K.K. Chaubey, S. Gupta, K.D. Rawat, A. Kumar, A.K. Bhatia, A.K. Srivastav, K. Dhama, Bio-load and bio-type profiles of *Mycobacterium avium* subspecies *paratuberculosis* infection in the farm and farmer's herds / flocks of domestic livestock: a 28 years study (1985–2013), Transbound. Emerg. Dis. 61 (2014) 1–13.

[5] K.K. Chaubey, R.D. Gupta, S. Gupta, S.V. Singh, A.K. Bhatia, S. Jayaraman, N. Kumar, A. Goel, A.S. Rathore, J.S. Sahzad, B.J. Sohal, M. Stephen, M. Singh, K. Goyal, A. Dhama, Derakhshandeh, trends and advances in the diagnosis and control of paratuberculosis in domestic livestock, Vet. Q. 36 (2016) 203–227.

[6] K.K. Chaubey, S.V. Singh, S. Gupta, M. Singh, J.S. Sohal, N. Kumar, M.K. Singh, A.K. Bhatia, K. Dhama, *Mycobacterium avium* subsp. *paratuberculosis* - an important food borne pathogen of high public health significance with special reference to India: an update, Vet. Q. 37 (2017) 282–299.

[7] F. Beaudeau, M. Melliard, A. Joly, H. Seegers, Reduction in milk yield associated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in dairy cows, Vet. Res. 38 (2007) 625–634.

[8] A.B. Garcial, L. Shaloo, Invited review: the economic impact and control of paratuberculosis in cattle, J. Dairy Sci. 98 (2015) 5019–5039.

[9] K.D. Rawat, S. Chaudhary, N. Kumar, S. Gupta, K.K. Chaubey, S.V. Singh, K. Dhama, A.K. Verma, R. Deb, Economic losses in a commercial dairy farm due to the outbreak of Johne's disease in India, Res. J. Vet. Pract. 2 (2014) 73–77.

[10] O.R. Vinodhkumar, L. Gunaseelan, B.S. Ronald, S.M. Sakthivalan, Slaughter house prevalence of ovine paratuberculosis in southern India, Trop. Anim. Health Prod. 45 (2013) 1063–1069.

[11] A. Tiwari, J.A. Van Leeuwen, I.R. Dohoo, G.P. Keefe, A. Weersink, Estimate of the direct production losses in Canadian dairy herds with subclinical *Mycobacterium avium* subspecies *paratuberculosis* infection, Can. Vet. J. 49 (2008) 569–576.

[12] C. Kirkeby, K. Graesbøll, S. Nielsen, L. Christiansen, N. Toft, E. Rattenborg, T. Halasa, Simulating the epidemiological and economic impact of paratuberculosis control actions in dairy cattle, Front. Vet. Sci. 3 (2016) 90, https://doi.org/10.3389/fvets.2016.00090.

[13] R.W. Sweeney, Pathogenesis of paratuberculosis, Vet. Clin. N. Am. Food Anim. Pract. 27 (2011) 537–546.

[14] R.H. Whitlock, C. Buergelt, Preclinical and clinical manifestations of paratuberculosis (including pathology), Vet. Clin. N. Am. Food Anim. Pract. 12 (1996) 345–356.

[15] V. Maroudam, M. Subramanian, P. Kumar, D. Raj, Paratuberculosis: diagnostic methods and their constraints, J. Vet. Sci. Technol. 6 (2015) 5.

[16] R.J. Whittington, E.S.G. Sergeant, Progress towards understanding the spread, detection and control of *Mycobacterium* avium subsp. *paratuberculosis* in animal populations, Aust. Vet. J. 79 (2001) 267–278.

[17] A.V. Singh, S.V. Singh, D.K. Verma, R. Yadav, P.K. Singh, J.S. Sohal, Evaluation of "Indigenous absorbed ELISA Kit" for the estimation of seroprevalence of *mycobacterium avium* subspecies *paratuberculosis* antibodies in Human Beings in North India, ISRN Vet. Sci. 2011 (2011) 636038, , https://doi.org/10.5402/2011/636038.

[18] P.K. Singh, S.V. Singh, H. Kumar, J.S. Sohal, A.V. Singh, Diagnostic application of IS*900* PCR using blood as a source sample for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in early and subclinical cases of caprine paratuberculosis, Vet. Med. Int. 2010 (2010) 748621.

[19] F.F.A. Jesse, A.A. Bitrus, Abba Yusuf, M.A. Sadiq, E.L.T. Chung, I.U. Hambali, M.A.M. Lila, A.W. Haron, Clinical and gross pathological findings of Johne's disease in a calf: a case report, J. Adv. Vet. Anim. Res. 3 (3) (2016) 292–296.

[20] C.D. Buergelt, A.W. Layton, P.E. Ginn, M. Taylor, J.M. King, P.L. Habecker, E. Mauldin, R. Whitlock, C. Rossiter, M.T. Collins, The pathology of spontaneous paratuberculosis in the North American Bison (*Bison bison*), Vet. Pathol. 37 (2000) 428–438.

[21] N.P. Kurade, B.N. Tripathi, K. Rajukumar, N.S. Parihar, Sequential development of histologic lesions and their relationship with bacterial isolation, faecal shedding, and immune responses during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. *Paratuberculosis*, Vet. Pathol. 41 (2004) 378–387.

[22] J.D. Bancroft, A. Stevens, Theory and Practice of Histological Techniques, Churchill Livingston, Edinburgh, London, Melbourne and New York, 1990.

[23] E.B. Prophet, B. Mills, J.B. Arrington, L.H. Sobin, Laboratory Methods in Histopathology, Armed Force Institute of Pathology, Washington, D.C, 1994, pp. 21–119.

[24] N.Q. Hailat, W. Hananeh, A.S. Metekia, J.R. Stabe, S. Al-Majali, A. Lafi, Pathology of subclinical paratuberculosis (Johne's Disease) in Awasishep with reference to its occurrence in Jordan, Vet. Med. 5 (2010) 590–602.

[25] I. Sevilla, S.V. Singh, J.M. Garrido, G. Aduriz, S. Rodriguez, M.V. Geijo, R.J. Whittington, V. Saunders, R.H. Whitlock, R.A. Juste, Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions, Rev. Sci. Tech. 24 (2005) 1061.

[26] S.V. Singh, A.V. Singh, R. Singh, K.S. Sandhu, P.K. Singh, J.S. Sohal, V.K. Gupta, V.S. Vihan, Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India, Comp. Immunol. Microbiol. Infect. Dis. 30 (2007) 175.

[27] G.S. harma, S.V. Singh, I. Sevilla, A.V. Singh, R.J. Whittington, R.A. Juste, S. Kumar, V.K. Gupta, P.K. Singh, J.S. Sohal, V.S. Vihan, Evaluation of indigenous milk ELISA with m-culture and m-PCR for the diagnosis of bovine Johne's disease (BJD) in lactating Indian dairy cattle, Res. Vet. Sci. 84 (2008) 30.

[28] K.K. Chaubey, S.V. Singh, A.K. Bhatia, Evaluation of 'Recombinant secretary antigens' based 'Cocktail ELISA' for the diagnosis of Johne's disease and to differentiate non-infected, infected and vaccinated goats in combination with indigenous ELISA test, Small Rumin. Res. 165 (2018) 24–29.

[29] M.T. Collins, Interpretation of a commercial bovine paratuberculosis enzyme-linked immune-sorbent assay by using likelihood ratios, Clin. Vaccine Immunol. 9 (2002) 1367–1371.

[30] S.V. Singh, A.V. Singh, P.K. Singh, J.S. Sohal, N.P. Singh, Evaluation of an indigenous ELISA for diagnosis of Johne's disease and its comparison with commercial kits, Indian J. Microbiol. 47 (2007) 251.

[31] A.V. Singh, S.V. Singh, J.S. Sohal, P.K. Singh, Comparative potential of modified indigenous, indigenous and commercial ELISA kits for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* in goat and sheep, Indian J. Exp.Biol. 47 (2009) 379.

[32] O.P. Paliwal, B.S. Rajya, Evalution of paratuberculosis in goats: pathomorphological studies, Indian J. Vet. Pathol. 6 (1982) 29–34.

[33] K. Rajkumar, Pathology and Diagnosis of Caprine Paratuberculosis, M.V.Sc. Thesis Deemed University, IVRI, Izatnagar, 1998.

[34] S.K. Munjal, B.N. Tripathi, O.P. Paliwal, Progressive immunopathological changes during early stages of experimental infection of goats with *Mycobacterium avium* subspecies *paratuberculosis*, Vet. Pathol. 42 (2005) 427–436.

[35] W.M. Allen, S. Berrett, D.S. Patterson, A biochemical study of experimental Johne's disease. I. Plasma protein leakage into the intestine of sheep, J. Comp. Pathol. 84 (1974) 381–384.

[36] C.J. Clarke, D. Little, The pathology of ovine paratuberculosis: gross and histological changes in the intestine and other tissues, J. Comp. Pathol. 114 (1996) 419–437.

[37] C.J. Clarke, I.A. Patterson, K.E. Armstrong, J.C. Low, Comparison of the absorbed ELISA and agar gel immune-diffusion test with clinic-pathological findings in ovine clinical paratuberculosis, Vet. Rec. 139 (1996) 618–621.

[38] V. Perez, J.F. Garcia Marin, J.J. Badiola, Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep, J. Comp. Pathol. 114 (1996) 107–122.

[39] M.A. Al-Dubaib, O.M. Mahmoud, Paratuberculosis of goats at Qassim region of central Saudi Arabia, Bulg. J. Vet. Med. 11 (2008) 65–69.

[40] N.B. Harris, R.G. Barletta, *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine, Clin. Microbiol. Rev. 14 (2001) 489–512.

[41] Y. Yokomizo, R.S. Merkal, P.A. Lyle, Enzyme-linked immunosorbent assay for-detection of bovine immunoglobulin G1 antibody to a protoplasmic antigen of

Mycobacterium paratuberculosis, Am. J. Vet. Res. 44 (1983) 2205–2207.
[42] J.C. Cox, D.P. Drane, S.L. Jones, S. Ridge, A.R. Milner, Development and evaluation of a rapid absorbed enzyme immunoassay test for diagnosis for Johne's disease in cattle, Aust. Vet. J. 68 (1991) 157–160.
[43] M.P. Reichel, R. Kittelberger, M.E. Penrose, R.M. Meynell, D. Cousins, T. Ellis, L.M. Mutharia, E.A. Sugden, A. Johns, G.W. de Lisle, Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *Paratuberculosis* infection in cattle and analysis of the antigens involved, Vet. Microbiol. 66 (1999) 135–150.
[44] S. Eda, J.P. Bannantine, W.R. Waters, Y. Mori, R.H. Whitlock, M.C. Scott, C.A. Speer, A highly sensitive and subspecies specific surface antigen enzymes-linked immunsorbent assay for diagnosis of Johne's disease, Clin. Vaccine Immunol. 13 (2006) 837–844.
[45] K.K. Chaubey, S.V. Singh, S. Gupta, S. Jayaraman, M. Singh, B.J. Stephan, K.D. Rawat, A. Pachoori, K. Dhama, A.K. Bhatia, Diagnostic potential of three antigens from geographically different regions of the world for the diagnosis of Ovine Johne's disease in India, Asian J. Anim. Vet. Adv. 10 (2015) 567–576.
[46] R.S. Sweeney, R.H. Whitlock, C.L. Buckley, Evaluation of commercial enzyme-linked immunosorbent assay for diagnosis of paratuberculosis in dairy cattle, J. Vet. Diagn. Invest. (1995) 488–493.
[47] S.S. Nielsen, N. Toft, Ante mortem diagnosis of paratuberculosis: a review of accuracies o ELISA, interferon-gamma assay and faecal culture techniques, Vet. Microbiol. 129 (2008) 217–235.
[48] S.V. Singh, Diagnosis of Paratuberculosis in Goats, [PhD dissertation] CSA University of Agriculture & Technology, Kanpur, 1998.
[49] K. Kathaperumal, V. Kumanan, S. McDonough, L.H. Chen, S.U. Park, M.A.S. Moreira, B. Akey, J. Huntley, C.F. Chang, Y.F. Chang, Evaluation of immune responses and protective efficacy in a goat model following immunization with a cocktail of recombinant antigens and a polyprotein of*Mycobacterium avium* subsp. *paratuberculosis*, Vaccine 27 (2009) 123–135.
[50] T.J. Radosevich, T.A. Reinhardt, J.D. Lippolis, J.P. Bannantine, J.R. Stabel, Proteome and differential expression analysis of membrane and cytosolic proteins from*Mycobacterium avium*subsp. *paratuberculosis* strains K-10 and 187, J. Bacteriol. 189 (2007) 109–1117.
[51] M.L. Mon, M. Viale, G. Baschetti, F.A. Pinedo, A. Gioffre, T. Gabriel, P. Willemsen, D. Bakker, M.I. Romano, Search for *Mycobacterium avium* subspecies *paratuberculosis* antigens for the diagnosis of paratuberculosis, Vet. Med. Int. 2012 (2012) 1–9.
[52] D. Pahangchopi, R.V. Singh, S.V. Singh, P. Das, D. Sharma, T. Sardana, N. Kumar, K.K. Chaubey, S. Gupta, Evaluation of 'cattle' and 'Indian Bison' type antigens of *Mycobacterium avium* subspecies *paratuberculosis* for diagnosis of Bovine Johne's Disease using 'Indigenous ELISA' and AGPT, Indian J. Exp. Biol. 52 (2014) 1182.
[53] P.K. Singh, S.V. Singh, A.V. Singh, J.S. Sohal, Screening of tissues and serum by culture, PCR and ELISA for the detection of *Mycobacterium avium* subspecies *paratuberculosis* from cases of clinical ovine Johne's disease in farmer's flocks, Ind. J. Anim. Sci. 78 (2008) 1052–1056.
[54] A. Kumar, S.V. Singh, A.K. Srivastava, N.K. Gangwar, P.K. Singh, S. Gupta, K.K. Chaubey, R. Tiwari, S. Chakraborty, K. Dhama, Comparative evaluation of 'Indigenous' and commercial vaccines in double challenge model for the control of caprine paratuberculosis in India, J. Biol. Sci. 14 (2014) 169–182.
[55] D. Yadav, S.V. Singh, A.V. Singh, I. Sevilla, R.A. Juste, P.K. Singh, J.S. Sohal, Pathogenic 'bison type' *Mycobacterium avium* subspecies *paratuberculosis* genotype characterized from riverine buffalo (*Bubalus bubalis*) in North India, Comp. Immunol. Microbiol. Infect. Dis. 31 (2008) 373.
[56] V. Kumar, A.K. Bhatia, S.V. Singh, Evaluation of efficacy of the species specific antigens in the diagnosis of ovine and caprine Paratuberculosis using plate ELISA, J. Immunol. Immunopathol. 8 (2006) 48.
[57] K.K. Chaubey, S.V. Singh, A.K. Bhatia, Comparison of 'Indigenous ELISA kit' with 'Ethanol Vortex ELISA kit (USA)' for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle population in 'Reverse Ice-burg' environment: in search of a 'globally relevant kit', Indian J. Exp. Biol. 56 (2018) 279–286.
[58] K.K. Chaubey, S.V. Singh, A.K. Bhatia, R.D. Gupta, S. Gupta, A. Varshney, M. Singh, M.K. Singh, Z. Hemati, A.S. Rathore, Detection limits of recombinant secretary proteins versus semipurified protoplasmic antigens for the diagnosis of spontaneous cases of *Mycobacterium avium* subspecies *paratuberculosis* infection in domestic ruminants, J. Vet. Sci. Technol. 9 (2018) 552.
[59] J.P. Bannantine, J.R. Stabel, E.A. Lamont, R.E. Briggs, S. Sreevatsan, Monoclonal antibodies bind a SNP-sensitive epitope that is present uniquely in *Mycobacterium avium* subspecies *paratuberculosis*, Front. Microbiol. 2 (2011) 1–13.
[60] M. Malamo, Y. Sakoda, H. Ozaki, H. Kida, Development of ELISA to detect antibodies specific to *Mycobacterium avium*subsp. *Paratuberculosis* with truncated 34 kDa proteins, Jpn. J. Vet. Res. 54 (2006) 99–107.
[61] S.J. Shin, H.S. Yoo, S.P. McDonough, Y.F. Chang, Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *Mycobacterium avium* subsp. *Paratuberculosis*, J. Vet. Sci. 5 (2004) 111–117.
[62] D. Cho, S.J. Shin, A.M. Talaat, M.T. Collins, Cloning, expression, purification and sero-diagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins, Protein Expr. Purif. 53 (2007) 411–420.
[63] J.P. Bannantine, D.O. Bayles, W.R. Waters, M.V. Palmer, J.R. Stabel, M.L. Paustian, Early antibody response against *Mycobacterium avium* subspecies *Para-tuberculosis* antigens in subclinical cattle, Proteome Sci. 6 (2008) 5.
[64] K.M. Samanich, M.A. Keen, V.D. Vissa, J.D. Harder, J.S. Spencer, J.T. Belisle, S. Zolla-Pazner, S. Laal, Sero-diagnostic potential of culture filtrate antigens of Mycobacterium tuberculosis, Clin. Diagn. Lab. Immunol. 7 (2000) 662–668.
[65] W.R. Waters, M.V. Palmer, T.C. Thacker, J.P. Bannantine, H.M. Vordermeier, R.G. Hewingson, R. Greenwald, J. Esfandiari, J. McNair, J.M. Pollock, P. Andesen, K.P. Lyashchenko, Early antibody responses to experimental *Mycobacterium bovis* infection of cattle, Clin. Vaccine Immunol. 13 (2006) 648–654.
[66] S.J. Shin, D. Cho, M.T. Collins, Diagnosis of bovine paratuberculosisby a novel enzyme-linked immunosorbent assay based on early secreted antigens of *Mycobacterium avium* subsp. *Paratuberculosis*, Clin. Vaccine Immunol. 15 (2008) 1277–1281.
[67] S. Gumber, G. Eamens, R.J. Whittington, Evaluation of a pourquier ELISA kit in relation to agar gel immuno-diffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without *Mycobacterium paratuberculosis* infection, Vet. Microbiol. 115 (2006) 91–101.
[68] S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal, M.C. Sharma, Therapeutic effects of a new "Indigenous Vaccine" developed using novel native "Indian Bison type" genotype of *Mycobacterium avium* subspecies *paratuberculosis* for the control of clinical Johne's disease in naturally infected goat herds in India, Vet. Med. Int. 2010 (2010) 351846.
[69] P.T. Willemsen, J. Westerveen, A. Dinkla, D. Bakker, F.G. Zijderveld, J.E. Thole, Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominentimmune targets, Vet. Microbiol. 114 (2010) 337–344.
[70] M. Pradenas, M.C. Jara, N. Hernandez, A. Zambrano, M.T. Collins, J. Kruze, Antibody recognition to secreted proteins of *Mycobacterium avium* subsp. *Paratuberculosis*in sera from infected ruminants, J. Vet. Microbiol. 138 (2009) 378–383.
[71] V. Dheenadhayalan, K.S. Shin, C.F. Chang, C.D. Chang, S.J. Wang, S. McDonough, Cloning and characterization of the genes coding for antigen 85A, 85B and 85C of Mycobacterium avium subsp. Paratuberculosis, DNA Seq. 13 (2002) 287–294.