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Detection Limits of Recombinant Secretary Proteins versus Semi-purified Protoplasmic Antigens for the Diagnosis of Spontaneous Cases of *Mycobacterium avium* subspecies *paratuberculosis* Infection in Domestic Ruminants

Kundan Kumar Chaubey[1,2], Shoor Vir Singh[1*], Ashok Kumar Bhatia[2], Rinkoo Devi Gupta[3], Saurabh Gupta[1], Ankur Varshney[4], Manju Singh[1], Manoj Kumar Singh[1], Zahra Hemati[5] and Abhishek Singh Rathore[3]

[1]*Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO-Farah, Mathura, Uttar Pradesh, India*

[2]*Department of Microbiology and Immunology, GLA University, Chaumuhan, Mathura, Uttar Pradesh, India*

[3]*Department of Life Sciences and Biotechnology, South Asian University, New Delhi, India*

[4]*Department of Biotechnology, Mangla Yatan University, Aligarh, Uttar Pradesh, India*

[5]*Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

[*]**Corresponding author:** Shoor Vir Singh, Animal Health Division, Central Institute for Research on Goats, Makhdoom, PO-Farah, Mathura, Uttar Pradesh, India, Tel: +91 565 2763260; Fax: +91 565 2763246; E-mail: shoorvir.singh@gmail.com

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Abstract

Johne's disease is world-wide in distribution and in the absence of control programs bio-load of *Mycobacterium avium* subspecies *paratuberculosis* continues to increase in domestic livestock and their milk in the country. For the diagnosis of MAP we developed novel recombinant secretary proteins based 'cocktail ELISA' by cloning using six highly immunogenic secretary proteins. C ELISA was compared with extensively validated 'indigenous ELISA kit' using semi-purified protoplasmic antigens from novel and native strains of MAP (Indian Bison Type). A total of 1301 serum from domestic livestock (726 goats; 150 sheep; 300 cattle; 125 buffaloes) were screened to estimate the bio-load of MAP. Using I ELISA bio-load of MAP was 55.3, 57.3, 58.0 and 44.0%. In C ELISA it was 54.6, 56.6, 55.6 and 42.4% in goats, sheep, cattle and buffaloes respectively. Two tests were comparable in detecting MAP infection, however C ELISA had better profiling of samples and highly specific. Bio-load in C ELISA was lower, since it lacked proteins against all stages of disease. Still using fewer proteins the loss in sensitivity was not much. Therefore, results of screening of field samples with unknown status and in different stages of disease, since MAP is endemic, support the use of C ELISA as future diagnostics for MAP infection in all the domestic livestock species. As test kit C ELISA will have more consistency in results due to use of expressed and purified six known proteins as compared to sPPA used in I ELISA.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; Recombinant secretary proteins; sPPA; Cocktail ELISA; Indigenous ELISA; Domesticated ruminants

Introduction

Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a notifiable disease and in absence of priority, bio-load of MAP continues to increase in domestic livestock and their milk in India as per our study of past 33 years [1]. MAP has widest host range, hence besides domestic livestock; MAP has been reported from wild ruminants, other animal species, primates and human beings [2,3]. Bio-load has also been reported from milk and milk products [1,2,4-13] and environment (soil and river water) samples [14]. In absence of control programs, bio-load of MAP sharply increased in animals [1,15] in milk and milk products and in human population [1,16] in India. JD seriously impedes and has negative impact on health and productivity of animals [17,18]. Johne's disease though list B disease in OIE classifications and invites trade restrictions globally. In India, JD is endemic and responsible for high morbidity and loss in animal productivity. Though, India is highest milk producer in the world, on the basis of number of animals. However, per animal productivity is very low even in Asian countries. Population of domestic livestock in India is highest (goats-133.0, sheep-63.0, cattle-187.0 and buffaloes-110.0 million) [19] in the world. But there is sharp upsurge in number of un-productive ruminants, primarily due to JD. Transmission of MAP continues through colostrum, milk, semen, pasture, soil and water [20-22], recently reported high bio-load of MAP in domestic livestock (42.7, 35.6, 22.5 and 40.9% in cattle, buffaloes, goats and sheep, respectively) and were 30.8% using multiple tests (microscopy, culture, PCR and I ELISA).

Control of JD was un-successful due to variable sensitivity and specificity of diagnostic tests in identifying MAP infected animals in early stages of infection [23]. MAP genome has 4350 protein-coding sequences including 3223 conserved proteins with known functions and 1088 conserved proteins with un-known functions with 39 proteins with unique identity [24]. Secretary antigens are reported to be highly immunogenic (immune-dominant) due to their presence in extracellular environment wherein likely encounter with sensitized immune cells was high. Cho et al. compared secretary antigens from supernatants and antigens of intracellular origin [25]. Secretary proteins un-like cellular proteins had greater zero-reactivity in MAP infected animals [26]. Secretary proteins are in focus for development of DIVA test and a marker vaccine. A range of new immunologically important secretary antigens of MAP have been identified using 2D-

gel electrophoresis, chromatography, mass spectrometry and peptide mass fingerprint [27]. Currently crude antigen mix of MAP is used for immune-diagnosis. Detailed categorization of antigen framework of MAP is important to develop improved diagnostics. Cho et al. [26] identified 14 secretary proteins of MAP-JTC303 by immune-blot and mass spectrometry. Polyclonal rabbit antibodies (Rabbit anti-MAP JTC303) applied were directed against these 14 proteins but it reacted only with five secretary proteins (MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep A (MAP Pep AN and MAP Pep AC). MAP 1693c is a Peptidyl-prolyl cis-trans isomerase domain-containing secreted protein having 18.3 kDa molecular weight (MW) conserved in MAP having unknown localization [27]. Another protein used in this study is MAP 2168c is a hypothetical protein expected 15.9 kDa MW protein having unknown functions and localization [25]. Mycobacterial ModD is an alanine and proline-rich secretory protein, expected 45-47 kDa MW immunogenic glycoprotein belonging to a fibronectin attachment protein (FAP) [26,27]. FAPs are a family of fibronectin-binding glycoproteins conserved in several mycobacterial species (*M. tuberculosis, M. bovis, M. vaccae, M. avium,* and *M. leprae*) [28,29]. FAPs are important for internalization and invasion of epithelial cells by MAP, and deletion of this gene leads to enhanced aggregation in *Mycobacterium smegmatis* [30]. Ag 85 complex is a 30-32 kDa family of three proteins (Ag85A, Ag85B and Ag85C) [31] each of which possesses an enzymatic mycolyl-transferase activity required for biogenesis of cord factor (trehalose-dimycolate). Proteins are encoded by three paralogous genes (fbpA, fbpB and fbpC) located in distinct regions of bacterial genome [32]. Genes encoding three Ag 85 components from MAP have been sequenced, and at protein level, a 99% sequence identity with *M. avium* was found, with a single amino acid residue difference for each protein [33] isoleucine/threonine in position 284 of the mature Ag85C protein in MAP and *M. avium*, respectively. Comparisons have been made between mature protein sequences of MAP Ag 85 and *M. bovis* Ag 85. The 87% of amino acid sequences of 85C protein were identical between MAP (MAP 3531c) and *M. bovis* [33]. MAP PepA is a serine protease and has a putative signal sequence at N-terminus. Native PepA was significantly more antigenic in infected sheep, goats and deer [34]. PepA is conserved in *Mycobacterium* spp, e.g., a virulent H37Ra strain, virulent Erdman strain, H37Rv strain, and clinical isolate CSU93 with 100% identity [26].

Serological tests have been frequently used in screening, sero-survey and diagnosis of MAP infection in domestic livestock [35]. Indigenous ELISA (I ELISA) we developed and first time made indigenous kit [36] for screening of serum and milk of 4 domestic livestock species and also for diagnosis of MAP infection in human population, since 2007 [1,15,37,38]. In this study, we identified six (MAP 1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN, MAP Pep AC) secretary proteins from the literature [26], were cloned, expressed, purified and used to develop cocktail_ELISA (C ELISA). New C ELISA was compared with I ELISA kit; using semi purified protoplasmic antigens (sPPA) of novel MAP strain 'S5' biotype ('Indian Bison Type') of goat origin for screening of goats, sheep, cattle and buffaloes against MAP infection.

Materials and Methods

History and collection of samples

Serum samples were collected from domestic livestock (Goats, sheep, cattle and buffaloes) suspected for MAP infection.

Sn	Livestock farms and place	Serum samples (n)	i_ELISA Positives, n (%)	c_ELISA Positives, n (%)
Goats				
1	CIRG, Makhdoom, Mathura, UP	175	118 (67.4)	116 (66.2)
2	Kurkunda village, Mathura, UP	60	35 (58.3)	35 (58.3)
3	Kharkhari village, Gurgaon, Haryana	174	72 (41.3)	71 (40.8)
4	Veterinary college, Mhow, MP	171	95 (23.3)	93 (54.3)
5	SADIL, Jahangirabad, Bhopal, MP	91	70 (76.9)	70 (76.9)
6	IGFRI, Jhansi, UP	40	7 (17.5)	7 (17.5)
7	Keetham village, Keetham, Agra, UP	15	5 (33.3)	5 (33.3)
Sub-total A		**726**	**402 (55.3)**	**397 (54.6)**
Sheep				
8	Bhai village, Mathura, UP	15	6 (40.0)	6 (40.0)
9	Keetham village, Keetham, Agra, UP	15	5 (33.3)	5 (33.3)
10	SKUAST-K, Srinagar, J & K	40	7 (17.5)	7 (17.5)
11	IGFRI, Jhansi, UP	40	28 (70.0)	27 (67.5)
12	LRIC, KVAFSU, Bidar, Karnataka	40	40 (100.0)	40 (100.0)
Sub-total B		**150**	**86 (57.3)**	**85 (56.6)**
Cattle				
13	Veterinary college, Mhow, MP	171	81 (47.3)	77 (45.0)
14	Kharkhari village, Gurgaon, Haryana	15	11 (73.3)	11 (73.3)
15	Yadu dairy farm, Alwar, Rajasthan	19	17 (89.4)	17 (89.4)
16	Gazipur, New Delhi	5	5 (100.0)	5 (100.0)
17	VCRI, Namakkal, Chennai, Tamil Nadu	75	53 (70.6)	50 (66.6)
19	Harewali, Narela, New Delhi	15	7 (46.6)	7 (46.6)
Sub-total C		**300**	**174 (58.0)**	**167 (55.6)**
Buffaloes				
20	Veterinary college, Mhow, MP	101	31 (30.6)	29 (28.7)
21	Jind, Haryana	24	24 (100.0)	24 (100.0)
Sub-total D		**125**	**55 (44.0)**	**53 (42.4)**

Table 1: Profile of domestic livestock species and the farms screened. n- Number; UP- Uttar Pradesh; MP- Madhya Pradesh; J & K- Jammu and Kashmir; IGFRI- Indian Grassland and Fodder Research Institute; VCRI-Veterinary College and Research Institute; LRIC- Livestock Research and Information Center; KVAFSU- Karnataka Veterinary Animal and Fishery Sciences University; CIRG- Central Institute for Research on Goats; SADIL- State Animal Disease Investigation Laboratory.

Since it is endemic in native livestock breeding farms and were driven from different states (Uttar Pradesh, Haryana, Madhya Pradesh, Tamil Nadu, Karnataka, Rajasthan) of the country (Table 1). Serum from 726 goats, 150 sheep, 300 cattle and 125 buffaloes were collected or received for screening against MAP and stored at -20°C until used.

Brief procedure of cloning, expression and purification of RSPs

Easy cloning pJET 1.2/blunt vector (Clone-JET PCR Cloning kit, Cat. no.: #K1231, Thermo fisher Scientific), pTZ57R/T Cloning vector (Insta clone PCR Cloning Kit, Cat. no.: #1214, Thermo fisher Scientific, USA) and expression vector, pET-28a (+) and pET-22b (+) were used. Both expression vectors contained a T7 promoter and C-terminal 6X His-tag coding sequence of the multiple cloning regions. Six secretary proteins (MAP 1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN, MAP Pep AC) were selected. Primers to six genes (MAP 1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN, MAP Pep AC) of corresponding six proteins were designed (Primer Quest tool and Oligo analyzer 3.1, Integrated DNA Technology, USA) using sequences from MAP K10 (cattle type) bio-type and were amplified by designed primers from novel native MAP strain 'S5' genomic DNA. However, one gene (MAP ModD) could not be cloned in pET 28a expression vector, therefore, this gene (MAP ModD) was cloned afresh in pET 22b (+) expression vector, using primers [26]. Purification of PCR products was done using gel extraction purification kit (Gene Jet plasmid mini prep kit, Cat. No. K0502, Thermo fisher scientific, USA). Purified PCR products ligated with pJET 1.2/blunt vector or pTZ57R/T cloning vector as per protocols. Recombinant clones were transformed into competent *E. coli* XL-1/XL-10/DH5α. After sequencing the inserted ORFs, fragments were cloned into sites for directional ligation into pET-28a (+) or, pET-22b (+) vectors after digestion with specific restriction enzymes. Ligation products then transformed into *E. coli* Rosetta/Bl-21 host cells for expression. Each transformed *E. coli* clone was grown in LB medium containing specific antibiotics and expression of cloned proteins were induced for 18-24 h by adding IPTG to optimized concentration range (0.3 mM to 1 mM). Cells were harvested by centrifugation and proteins were extracted by first disrupting cells by sonication in equilibration buffer (50 mM, Tris. HCl, 200 mM NaCl, 5 mm DTT, 1 mM PMSF, pH 8.0). Cellular debris was removed by centrifugation and supernatant saved. Recombinant proteins containing a C-terminal histidine tag were purified using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Soluble proteins extracts were added to Ni-NTA resin previously washed with sonication buffer. Protein binding was occurred by gentle agitation for 4-6 h at 4°C. Proteins bound to resin were purified by Gravity flow Ni-NTA column. Fractions containing recombinant antigens were pooled dialyzed against sonication buffer. Recombinant proteins were concentrated and their protein was estimated by Bradford protein estimation assay and six recombinant secretary proteins based Cocktail-ELISA was optimized.

Recombinant secretary antigens (RSAs) based cocktail ELISA

'Indirect ELISA' was developed to detect IgG antibodies against cocktail of six recombinant antigens/proteins (MAP 1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN, MAP Pep AC) [39]. Flat bottom 96-well micro-titer plates (Catalogue no. 655061, Greiner bio-one, made in Germany) were coated with 100 µl of cocktail of recombinant secretary proteins containing 1 µg of each of 6 antigens diluted in 10 ml antigen coating buffer. Coated plates were incubated over-night at 4°C. After incubation, antigen-coated plates were washed one time with washing buffer (1X PBS containing 0.05% [v/v] Tween-20). Un-coated surfaces were then blocked (100 µl/well) with blocking buffer (PBS containing 5% skimmed milk) for one hour at 37°C. Following three washes with washing buffer, 100 µl of diluted serum (1:50) in serum dilution buffer (0.2 gm BSA in 20 ml of 1X PBS containing 0.05% [v/v] Tween-20) were added in duplicate to each well. Plates were incubated at 37°C for two hours, emptied and washed four times with washing buffer. Secondary antibodies used in this assay were peroxidase-labeled anti-species whole IgG antibody produced in rabbit at the dilution of 1:5000 for goats and sheep; 1:4000 for cattle and buffaloes in 1 X PBS. 100 µl of secondary anti-species antibody was added to each well and incubated for 50 minutes at 37°C. After four times of washing, 100 µl of chromogenic substrate solution of O-Phenylenediamine dihydrochloride (OPD) (Cat. No. P3804, Sigma-Aldrich, Inc) prepared as per manufacturer's recommendation was added to each well. Plates were incubated for 10-15 minutes in the dark at 37°C. Extent of the color development (Optical density) was measured at the absorbance of 450 nm using Bio-RAD i mark ELISA plate reader. Serum samples from culture positive and negative animals were used as positive and negative controls respectively.

Interpretation: Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per Collins (2002).

S/P percent=[(Sample OD-Negative OD)/(Positive OD-Negative OD)].

S/P ratios and corresponding status of recombinant cocktail secretary proteins based ELISA in animals was determined (Table 2).

S. no	S/P Ratio	Johne's disease status
1	0.00-0.09	Negative (N)
2	0.10-0.24	Suspected or Borderline (S)
3	0.25-0.39	Low Positive (LP)
4	0.4-0.99	Positive (P)
5	1.0-10.0	Strong Positive (SP)

Table 2: S/P ratios and status of Johne's disease, *LP+S+N- Negative for MAP; SP+P- Positive for MAP.

Native antigens: For comparison semi-purified Protoplasmic antigens (sPPA) prepared from native novel biotype ('Indian Bison Type') of MAP strain 'S5' recovered from an advance case of JD in a goat were used [40]. Strain recovered from this terminally sick Jamunapari goat (extremely weak and recumbent) located at Central Institute for Research on Goats (CIRG), Makhdoom, which later succumbed to disease (JD) [5]. Protoplasmic Antigen (PPA) was harvested by whole cell sonication and lysate was centrifuged and supernatant was collected (semi-purified).

Indigenous ELISA kit: Indigenous ELISA (I ELISA) kit was initially developed for the screening of goats and sheep has since been standardized for the screening of cattle and buffaloes. Antigens (sPPA) from 'Indian Bison Type' MAP were standardized at 0.1 micro-gram (µg) per well of the micro-titer plate. Serum samples were used in 1:50 dilution and anti-species horse radish peroxidase conjugate (Sigma Aldrich, USA) at the dilution of 1:5000 for goats and sheep; 1:4000 for cattle and buffaloes in 1X PBS. Serum samples from culture positive

and negative animals and human beings were used as positive and negative controls, respectively.

S/P ratio: Optical densities (OD) were expressed as sample-to-positive (S/P) ratios as per Collins (2002) by following calculations (Table 2).

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

S/P ratio and corresponding status of JD in animals: Table 2 depicts the sample to positive ratios (S/P) and status of Johne's disease in the host.

Sensitivity and Specificity:

Sensitivity=True Positive × 100/True Positive+False Negative

Specificity=True Negative × 100/True Negative+False Positive

Statistical analysis

Mc Nemar's test and kappa agreement have applied for the measure the statistical significance between results of two tests (GraphPad software, USA).

Results

Serum ELISA in particular has been regarded as fast screening tests for 'mass screening' of large population of domestic livestock. Serum samples of 726 goats, 150 sheep, 300 cattle and 125 buffaloes were screened using two ELISA tests, Using I ELISA and C ELISA, the bio-load of MAP was 55.3, 57.3, 58.0 and 44.0% and 54.6, 56.6, 55.6 and 42.4% in goats, sheep, cattle, buffaloes and humans, respectively (Figure 1, Table 3).

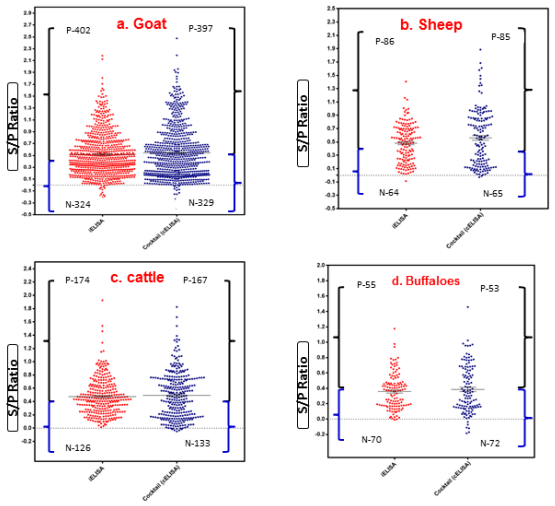

Figure 1: Scattered plot of c_ELISA showing range of correlations with i_ELISA in four domestic livestock species (Goats-726; Sheep-150; Cattle-300; Buffaloes-125).

Species	Tests	Positives, n (%)			Negatives, n (%)			
		SP	P	Total Pos.	LP	S	N	Total Neg.
Goats (726)	i_ELISA	76 (10.4)	326 (44.9)	402 (55.3)	149 (20.5)	117 (16.1)	58 (7.9)	324 (44.6)
	c_ELISA	113 (15.5)	284 (39.1)	397 (54.6)	87 (11.9)	148 (20.3)	94 (12.9)	329 (45.3)
Sheep (150)	i_ELISA	4 (2.6)	82 (54.6)	86 (57.3)	22 (14.6)	26 (17.3)	16 (10.6)	64 (42.6)
	c_ELISA	18 (12.0)	67 (44.6)	85 (56.6)	11 (7.3)	32 (21.3)	22 (14.6)	65 (43.3)
Cattle (300)	i_ELISA	10 (5.7)	164 (54.6)	174 (58.0)	58 (19.3)	55 (18.3)	13 (4.3)	126 (42.0)
	c_ELISA	20 (6.6)	147 (49.0)	167 (55.6)	34 (11.3)	64 (21.3)	35 (11.6)	133 (44.3)
Buffaloes (125)	i_ELISA	1 (0.8)	54 (43.2)	55 (44.0)	20 (16.0)	37 (29.6)	13 (10.4)	70 (56.0)
	c_ELISA	2 (1.6)	51 (40.8)	53 (42.4)	17 (13.6)	35 (28.0)	20 (16.0)	72 (57.6)

Table 3: Comparison of I ELISA and C ELISA values for the screening of domestic livestock and human beings against MAP infection. *Values in parentheses are percent; LP- low positive, S-suspected, N-negative, P-positive, SP-Strong positive for MAP infection. (LP+S+N)-Negative for MAP; SP+P-Positive for MAP or infected.

Goats: Of 726 serum samples from different breeds of goats and from different places of country (Table 1) were screened by I ELISA and C ELISA abd 402 (55.3%) and 397 (54.6%) goats were positive and 324 (44.6%) and 329 (45.3%) were negative, respectively (Table 3). Comparison of zero-reactivity in C ELISA with I ELISA showed that results of positive and negative goats were comparable, 10 (1.37%) positive samples in I ELISA were missed by C ELISA and 5(0.68%) samples positive in C ELISA were missed by I ELISA (Table 4). I ELISA was more sensitive in two tests while C ELISA is more specific than I ELISA.

I ELISA n (%)		C ELISA, n (%)					
		SP	P	LP	S	N	Total
SP	**76 (10.4)**	46 (6.3)	25 (3.4)	5 (0.68)	-	-	**76 (10.4)**
P	**326 (44.9)**	67 (9.2)	254 (27.4)	4 (0.5)	1 (0.1)	-	**326 (44.9)**
LP	**149 (20.5)**	-	4 (0.5)	77 (10.6)	63 (8.6)	5 (0.68)	**149 (20.5)**

S	**117 (16.1)**	-	1 (0.1)	0 (0.0)	77 (10.6)	39 (5.3)	**117 (16.1)**
N	**58 (7.9)**	-	-	-	8 (1.1)	50 (6.8)	**58 (7.9)**

Table 4: Status of JD in I ELISA and C ELISA in goats. Total serum-726, LP-low positive, S-Suspected, N-Negative, P-Positive, SP-Strong, positive for MAP infection.

Sheep: Of 150 serum samples of sheep collected from 5 places in three states of country (Table 1). Screening by I ELISA and C ELISA, 86 (57.3%) and 85 (56.6%) sheep were positive and 64 (42.6) and 65 (43.3) were negative, respectively (Table 1). Approximately similar results were observed in positive and negative categories. One (0.66%) sheep positive in I ELISA was missed by C ELISA (Table 5). Like goats, here too, I ELISA was more sensitive and less specific as compared to C ELISA.

i_ELISA n (%)		c_ELISA, n (%)					
		SP	**P**	**LP**	**S**	**N**	**Total**
SP	**4 (2.6)**	4 (2.6)	-	-	-	-	**4 (2.6)**
P	**82 (54.6)**	14 (9.3)	67 (44.6)	1 (0.6)	-	-	**82 (54.6)**
LP	**22 (14.6)**	-	-	7 (4.6)	15 (10.0)	-	**22 (14.6)**
S	**26 (17.3)**	-	-	1 (0.6)	14 (9.3)	11 (7.3)	**26 (17.3)**
N	**16 (2.2)**	-	-	2 (1.3)	3 (2.0)	11 (7.3)	**16 (2.2)**

Table 5: Status of JD in I ELISA and C ELISA in sheep. Total serum-150, LP-low positive, S-suspected, N-negative, P-positive, SP-Strong positive for MAP infection.

Cattle: Cattle serum samples (300) were collected from 6 places in 5 states of country and screened by both ELISA tests (Table 1). On screening 174 (58.0) and 167 (55.6) cattle were positive and 126 (42.0%) and 133 (44.3%) were negative by I ELISA and C ELISA, respectively (Table 2). Comparable results were observed between zero-reactivity of I ELISA and C ELISA 7 (2.3%) positive cattle in I ELISA were missed by C ELISA (Table 6). Results showed high sensitivity and less specificity of I ELISA with respect to C ELISA.

I ELISA, n (%)		C ELISA, n (%)					
		SP	**P**	**LP**	**S**	**N**	**Total**
SP	**10 (3.3)**	4 (1.3)	5 (1.6)	-	1 (0.3)	-	**10 (3.3)**
P	**164 (54.6)**	16 (5.3)	142 (47.3)	6 (2.0)	-	-	**164 (54.6)**
LP	**58 (19.3)**	-	-	25 (8.3)	29 (9.3)	4 (1.3)	**58 (19.3)**
S	**55 (18.3)**	-	-	3 (1)	32 (10.6)	20 (6.6)	**55 (18.3)**
N	**13 (4.3)**	-	-	-	2 (0.6)	11 (3.6)	**13 (4.3)**

Table 6: Status of JD in I ELISA and C ELISA in cattle. Total serum-300, LP-low positive, S-suspected, N-negative, P-positive, SP-Strong positive for MAP infection.

Buffaloes: Serum samples of 125 buffaloes were collected from two places in two states of country. Buffaloes were screened for MAP infection by I ELISA and C ELISA and 55 (44.0%) and 53 (42.4%) were positive and 70 (56.0) and 72 (57.6%) were negative, respectively. Results were comparable in two ELISA tests. Two (1.6%) positive buffaloes in I ELISA were missed by C ELISA (Table 7). Results showed comparatively high specificity and less sensitivity of C ELISA than I ELISA in buffaloes also.

i_ELISA, n (%)		c_ELISA, n (%)					
		SP	**P**	**LP**	**S**	**N**	**Total**
SP	**1 (0.8)**	1 (0.8)	-	-	-	-	**1 (0.8)**
P	**54 (43.2)**	1 (0.8)	50 (40.0)	3 (2.4)	-	-	**54 (43.2)**
LP	**20 (16.0)**	-	-	13 (10.4)	6 (4.8)	1 (0.8)	**20 (16.0)**
S	**37 (29.6)**	-	-	2 (1.6)	26 (20.8)	9 (7.2)	**37 (29.6)**
N	**13 (10.4)**	-	-	-	3 (2.4)	10 (8.0)	**13 (10.4)**

Table 7: Status of JD in i_ELISA and c_ELISA in buffaloes. Total serum-125, LP-low positive, S-suspected, N-negative, P-positive, SP-Strong positive for MAP infection.

In Table 8, the results of I ELISA and C ELISA have been compared. Of 726 goats, 150 sheep, 300 cattle and 125 buffaloes screened 54.6% (397) goats, 56.6% (85) sheep, 55.6% (167) cattle, 42.0% (53) buffaloes and 44.6% (324) goats, 43.3% (65) sheep, 48.6% (146) cattle and 54.7% (69) buffaloes were positive and negative in both the tests. A small number of 0.68% (5) goats, 0.66 (1) sheep, 2.33 (7) cattle and 2.4% (3) buffaloes positive in I ELISA were missed in C ELISA test.

Tests	**Combinations, n (%)**			
	1	**2**	**3**	**4**
I ELISA	+	-	+	-
C ELISA	+	-	-	+
Goat (726)	397 (54.6)	324 (44.6)	5 (0.68)	0 (0.0)
Sheep (150)	85 (56.6)	65 (43.3)	1 (0.66)	0 (0.0)
Cattle (300)	167 (55.6)	146 (48.6)	7 (2.33)	0 (0.0)
Buffaloes (125)	53 (42.0)	69 (54.7)	3 (2.4)	0 (0.0)

Table 8: Comparative results of I ELISA and C ELISA in four domestic livestock species (Goats, sheep, cattle and buffaloes), *Value in parenthesis are per cent.

Sensitivity and specificity of C ELISA in domestic ruminants

a. Comparison of I ELISA with C ELISA: Sensitivity of I ELISA was 100.0% in all four domestic livestock species (goats, sheep, cattle, buffaloes). Specificity was 98.5, 98.5, 98.5 and 95.8% in goats, sheep, cattle, and buffaloes, respectively (Table 9).

b. Comparison of C ELISA with I ELISA: Sensitivity of C ELISA was 98.7, 98.8, 95.9 and 94.6% in goats, sheep, cattle, buffaloes, respectively and specificity was 100.0% in all four domesticated species (Table 9).

Species	Test	TP	TN	FP	FN	Sen and Sp %
Goats (726)	i_ELISA	397	324	5	0	100.0 and 98.5
	c_ELISA	397	324	0	5	98.7 and 100.0
Sheep (150)	i_ELISA	85	65	1	0	100 and 98.5
	c_ELISA	85	65	0	1	98.8 and 100.0
Cattle (300)	i_ELISA	167	146	7	0	100.0 and 98.5
	c_ELISA	167	146	0	7	95.9 and 100.0
Buffaloes (125)	i_ELISA	53	69	3	0	100.0 and 95.8
	c_ELISA	53	69	0	3	94.6 and 100.0

Table 9: Sensitivity and specificity of two types of antigens (Cocktail of recombinant secretary proteins and semi-purified protoplasmic) in c_ELISA with i_ELISA as standard test, TP- true positive; FP-false positive; TN- true negative; FN- false negative; Sen- sensitivity; Sp-specificity.

Statistical analysis

Using Mc-Nemar test and kappa agreement, P value and kappa agreement were calculated. P values were 0.0736, 1.000, 0.0233 and 0.2482 and Kappa agreements are 0.986, 0.987, 0.956 and 0.951 in goats, sheep, cattle and buffaloes, respectively. Strength of agreement was perfect in all the four domestic livestock species, respectively (Table 10).

S. no	Species	P value		Kappa	Strength of agreement	95% Confidence interval
		Status	Value			
1	Goats	Not quite significantly different	0.0736	0.986 ± 0.006	Perfect	0.974 to 0.998
2	Sheep	Difference is not significant	1.000	0.987 ± 0.013	Perfect	0.960 to 1.000
3	Cattle	Significantly different	0.0233	0.956 ± 0.016	Perfect	0.924 to 0.988
4	Buffaloes	Difference is not significant	0.2482	0.951 ± 0.028	Perfect	0.897 to 1.000

Table 10: P value (Mc-Nemar test) and Kappa agreement between I ELISA and C ELISA in goats, sheep, cattle, buffaloes,

Discussion

Control and eradication of MAP infection in domestic livestock is essential to prevent transmission to human population through food (milk) chain. Accurate and timely diagnostic is pre-requisite to achieve above goals. Therefore specificity of assay is crucial for screening of MAP infection in early stages of infection in domestic livestock species. In this study two 'indigenously developed ELISA' assays (I ELISA and C ELISA) were used for screening of goats, sheep, cattle and buffaloes. Johne's disease is a major health concern in domestic and wild animals as well as in human population [1,2,41]. Currently there is no test that can precisely diagnose all cases with 100% accuracy [14,42], therefore, we evaluated C ELISA with earlier developed and highly validated 'I ELISA' for better profiling of results and with view to improve the sensitivity and specificity of newly developed C ELISA assay.

'I ELISA kit' has been standardized for four domestic ruminant species [43,44]. Large number of studies compared the sensitivity and specificity of different ELISA assays for screening of MAP and to diagnose MAP infection in cattle and sheep. However studies are limited in goats and buffaloes being Asian animals. MAP is major threat for the health and productivity of domestic livestock. Country possessing highest population of buffaloes, goats (In India), cattle (I^{st}), sheep (III^{rd}) and leading milk producer in the world. But per animal productivity it is very low, even behind Egypt, Iran and Pakistan in Asian countries. This is mainly due to the fact that livestock population of the country is endemic for MAP infection [1,14]. In absence of control programs and focus, MAP infection continues to increase at fast rate in the domestic livestock population. Using semi-purified protoplasmic antigen (sPPA) of goat origin harvested from 'Indian Bison type' biotype of MAP (strain 'S5') based 'I ELISA' showed significantly higher sensitivity as compared to commercially available ELISA kits [40,43,45]. I ELISA correlated well with culture and was good screening test for domestic livestock [40]. In another study, sensitivity and specificity of I ELISA kit was 66.6, 75.0% and 68.1, 66.6% with tissue culture and PCR respectively [46]. Similar comparative studies showed that I ELISA had superior diagnostic potential exhibiting high sensitivity as compared to commercial ELISA kits [43]. Kumar et al. 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 [17]. Other studies also reported that I ELISA kit had improved detection rate of MAP in test samples [15,42,46] Using multiple tests including I ELISA, showed considerable rise in bio-load of MAP from 28.9% to 47.0% by Singh et al. While Yadav et al. used I ELISA for the screening of slaughtered buffaloes, 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' for MAP antibodies, respectively [47]. This study showed buffaloes being Asian domestic livestock species exhibited very low sensitivity. Kumar et al. indicated greater sensitivity of species specific antigens [46]. Chaubey et al. reported I ELISA (Indigenous 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. Pahangchopi et al. evaluated I ELISA used antigens from native MAP strain (S5), was superior over AGPT using commercial antigen (ATCC 19698, UK used for making Johnin) in India [48]. Chaubey et al. compared sensitivity and specificity of EV ELISA with I ELISA, EV ELISA was less sensitive than I ELISA [22]. These studies

showed major problem of lowered sensitivity of antigens used in commercial ELISA kits using different bio-type used as antigen source. 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 [49]. 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 [50-53].

Cho et al. 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 [27]. 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* [46,54-56]. Use of MAP CF antigens in ELISAs increased assay sensitivity by 25% over commercial ELISA's for low MAP shedding animals [57]. Recently, early zero-diagnosis was addressed using experimentally infected calves and screening for antibody responses to a panel of 96 recombinant MAP antigens [58]. Antibody responses were detected as early as 70 days post-infection. However, fluctuations in antibody responses and epitope specificity were observed over 321 days [59]. Similar results were found in our earlier studies [59], where in peak titers were achieved at 60 days after vaccination of goats. The variability in achieving peak titer varied as per the damage to the lymphoid system (mesenteric lymph nodes, Peyer's patches) in the infected animals [59]. In Indian conditions, animals in all the four livestock species are endemic for MAP infection [16], therefore normal condition of animals (physical and internal, especially lymphoid tissues) is 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 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 [25,60]. Pradenas et al. reported that most CF proteins have low molecular weight and reacted strongly with sera from culture-positive cases of JD [61]. 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 zero-diagnosis. JD. Dheenadhayalan et al. [36] also reported immunogenicity of five recombinant antigens, namely MAP 2411, ClpP (MAP 2281c), Ppa (MAP 0435 c), 990 MAP 0593 c and Gre A (MAP 1027 c). 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 1000 tested recombinant antigens of Ag85A, Ag85B, Ag85C and SOD in ELISA with sera from 60 MAP shedding cows and 22 non-shedding cows [15]. 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 S5 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.

Conclusion

Increased knowledge of the comparative accuracy of two ELISA based diagnostic assay may help to choose appropriate assay for the screening and control of MAP infection in the domestic livestock population of the country. Study demonstrated differences in the performance of the currently available diagnostic tests for *MAP* infection in goats, sheep, cattle and buffaloes. Indigenous ELISA was low-cost and easy to develop in laboratory had higher sensitivity in early infection of MAP and was good for screening test for four domestic livestock species. However, recombinant secretory proteins based cocktail ELISA showed better profiling of results with improved sensitivity and high specificity. So, cocktail of secretary proteins based C ELISA can be used as future diagnostics for MAP infection in domestic livestock species in India.

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 were performed under Indian Council of Medical Research Project [grant number 5/8/5/28/TF/2013/ECD-I], ICMR, New Delhi, India under reference number IAEC/CIRG/16-17 dated 12.05.2016 and confirmed that this project do not have any ethical issue. Serum samples 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.

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