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Recombinant secretory proteins based new 'Cocktail ELISA' as a marker assay to differentiate infected and vaccinated cows for *Mycobacterium avium* subspecies *paratuberculosis* infection

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Johne's disease is endemic in the domestic livestock population of India. Recently, we developed highly effective 'Indigenous vaccine' to control Johne's disease in animals. In order to gain disease free status as per World Organization for Animal Health, it is essential to have a marker assay to differentiate between infected and vaccinated animals before vaccine can be used in the field. We have developed a new marker assay 'Cocktail ELISA' using six 'recombinant secretary proteins' (MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN and MAP Pep AC) and evaluated for diagnosis of Johne's disease along with 'Indigenous ELISA kit'. This 'Cocktail ELISA' successfully differentiated the infected, vaccinated and healthy (non-infected) cows and will facilitate the use of Johne's disease vaccine to control the disease in cows at national levels.

Keywords: Cows, DIVA marker assay, Johne's disease, Livestock, MAP infection, Ns_PPA

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic granulomatous debilitating disease of domestic livestock leading to high morbidity and huge economic losses[1,2]. MAP is widely distributed in domestic and wild ruminants and also reported from rabbits, primates and human beings in India[3-7]. Due to high economic / production losses, World Organization for Animal Health (OIE) recommended restrictions in the international trade of JD infected animals. JD control and eradication program is severely hampered by limited knowledge of host factors controlling immune response to MAP[8] and unrecognized sub-clinical cases of MAP infection. 'Test and cull' approach has been frequently used in the developed countries[9] to stamp out the disease. In view of the failure of 'test and cull' methodology in local conditions here, we developed 'indigenous vaccine' against JD[10]. Inactivated/live attenuated JD vaccine are applied in many countries for a period of 5-35 years for controlling JD and were able to reduce JD incidence from 87.5 to 5%[10-12]. However, as per Office International des Epizooties (OIE), before 'indigenous vaccine' can be used in the field, it is essential to have a marker assay which can Differentiating Infected from Vaccinated Animals (DIVA). In the absence of DIVA assay, it is not possible to launch any control program using whole cell 'Indigenous vaccine'.

In developing DIVA marker assay, selection of antigens is a major challenge. Antigens should be pathogen specific for sensitive and specific detection of MAP. Cho *et al.*[13] identified fourteen proteins with potential diagnostic value for JD. They reported that secretory proteins/cultural filtrate proteins (CFPs) of MAP showed greater reactivity with MAP positive cows sera, with respect to number of antigens detected and the reaction intensity on western blots. These MAP CFPs are good antigen candidates for the development of improved sero-diagnostic tests for bovine JD. Use of CFPs increased sensitivity of ELISA by 25% over the commercial ELISAs for low MAP shedders in cows[14]. However, selection of antigens remains major challenge, since there is no single MAP-specific antigen that is recognized by all the stages of infected animals, especially those in early and sub-clinical stages of the disease.

Six recombinant secretory proteins (RSPs) of MAP were used to develop MAP based cocktail ELISA as 'marker assay', and their ability to differentiate infected and vaccinated animals.

Materials and Methods

Samples Profile

Faecal and serum samples were driven from cow available in 4 Gaushalas of the states [Rajasthan (RJ), Haryana (HR), Uttar Pradesh (UP) and Madhya Pradesh (MP) in 3 years (2013–2016). Four gaushalas volunteered to participate in the 'vaccine trial'. A total of 458 cows (82-Yadu dairy farm, Alwar, Rajasthan;

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279- Bhavika Dairy Farm, Gurgaoun, Haryana, 80-Vaishanav Gaushala, Bansi Vat, Vrindavan, UP and 17-Ekta Agronomic and livestock, Sundrel, MP) were included in this study. Physical condition of the cows (variable in age and mainly females) was mix type ≤50% cows were healthy with good physical conditions, 20-25% were in clinical to advance clinical stages of disease and were weak, debilitated and emaciated. All these cows were ear-tagged for identification. Of 458 cows in four herds, 178 were used in the study, out of which 112 cows were physically in good health and tested negative in faecal microscopy. About 66 cows that were weak and emaciated had clinical sign of JD, and poor physical conditions tested positive in faecal microscopy (Table 1). Of 112 healthy cows, 44 were vaccinated with 'Indigenous JD Vaccine' developed in India[15] and was under trial. The rest 68 cows in the healthy category were used as negative control (non-infected). Serum samples from categorised infected (66), vaccinated [6-12 months post vaccinated cows– (44)] and apparently healthy / non-infected cows (68) were kept at –20°C until use.

Shedding of MAP in feces (Microscopy)

Faecal samples were screened by microscopy (ZN staining) for shedding of MAP bacilli. The +4, +3 to +2 and +1 were taken as super, moderate and low shedders, respectively[4].

Cloning, expression and purification of RSPs

Six MAP immunogenic secretory proteins (MAP1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN and MAP Pep AC) were selected, Genes of respective recombinant secretory proteins (RSPs) were cloned and proteins were expressed and purified as per Chaubey *et al*.[15]. Proteins concentrations were estimated by Bradford protein estimation and were optimized for six recombinant antigens based 'cocktail ELISA'.

RSPs based cocktail ELISA (c_ELISA)

'Indirect ELISA' was developed to detect IgG antibodies against cocktail of the recombinant antigens/proteins (MAP1693c, MAP 2168c, MAP ModD, MAP 85c, MAP Pep AN, MAP Pep AC). Flat bottom 96-well microtiter plates (Catalogue no. 655061, Greiner bio-one, made in Germany) were coated with 100 µL of cocktail of recombinant secretory proteins containing 1µg 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). Uncoated 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 g 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 h, emptied and washed four times with washing buffer. Secondary antibodies used in this assay were peroxidase-labelled anti-bovine whole IgG antibody produced in rabbit at the dilution of 1:4000 in 1X PBS. About 100 µL of secondary antibovine antibody was added to each well and incubated for 50 min at 37°C. After four times 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 min in the dark at 37°C. Extent of the colour development (Optical density) was measured at the absorbance of 450 nm using Bio-RAD *i*-mark ELISA plate reader. Serum samples from infected, vaccinated/suspected and negative/healthy cows were used for the optimization of assay.

Table 1 — Screening of cows for JD using Microscopy (ZN staining)

Microscopy (ZN staining)		
Category	Numbers	Status of JD in cows
4+	2	Positive
3+	18	
2+	33	
1+	13	
N	112	Negative
Grand-total	178	

Interpretation

Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per Collins (2002)[16].

S/P Ratio= [(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 as Table 2:

Native antigens

For comparative analysis, native semi-purified Protoplasmic antigens (Ns_PPA) were prepared from the novel biotype ('Indian Bison Type') of MAP strain 'S 5' recovered from the advance case of JD in a goats[17]. This strain was isolated from a terminal case of JD (extremely weak and recumbent) in a

Table 2 — S/P ratios and corresponding status of cows with respect to JD using 'i_ELISA'

Calculated value of OD into S/P Ratio	Status of JD in cows*	Status of MAP infection in i_ELISA**
0.00-0.09	Negative (N)	Negative
0.10-0.24	Suspected or Borderline (S)	
0.25-0.39	Low Positive (LP)	
0.4-0.99	Positive (P)	Positive
1.0-10.0	Strong Positive (SP)	

[MAP infection S/P ratio categories: N, Negatives; S, Suspected; LP, Low positive; P, Positive; and SP, Strong positive for MAP infection, S+LP, Negative; and P+SP, Positive (Infected). *Adopted from Collin, 2002 in cows[22]; **As per Singh *et al*. in cows[4]]

Jamunapari goat located at Central Institute for Research on Goats (CIRG), Makhdoom, which later succumbed to JD.

Indigenous ELISA kit

Indigenous ELISA (i_ELISA) kit was developed for goats[18] and has since been standardized for the screening of cows[4]. Antigens (Ns_PPA) from 'Indian Bison Type' was standardized at 0.1 µg per well of the microtiter plate. Serum samples were used in 1:50 dilution and antibovine horseradish peroxidase conjugate (Sigma Aldrich, USA) in 1:4000 dilution. Serum samples from culture positive and negative cows were used as positive and negative controls, respectively.

Results & Discussion

Of 178 cows used for the study, 66 tested positive and 112 negative for *Mycobacterium avium paratuberculosis* (MAP) infection in faecal microscopy (Table 1). These 112 cows negative for MAP were split into two categories as follows: 44 for vaccination with "Indigenous JD Vaccine" developed in India[9] and the rest 68 healthy cows were taken as negative (non-infected). The 44 vaccinated cows were volunteered for the vaccine trial for 6-12 months post vaccination. Screening of 178 cows [in three categories *viz*., cows (i) having clinical sign, (ii) vaccinated, and (iii) apparently healthy with respect to MAP infection], revealed 110 (61.7%) and 66 (37.0%) were positive for MAP infection by i_ELISA and c_ELISA, respectively. Comparison of two ELISA tests showed that there was agreement of 75.2% (134) and mismatch (disagreement) of 24.7% (44) (Table 3).

For validation of c_ELISA as marker assay (DIVA), 178 categorized cows (66 infected, 68 healthy and 44 vaccinated) were screened by c_ELISA and i_ELISA. Screening of 44 vaccinated

Table 3 — Screening of non-infected, infected and vaccinated cows for JD using i_ELISA and c_ELISA tests

Tests	Combinations, n (%)				*n* (%)
	1	2	3	4	
i_ELISA	+	-	+	-	110 (61.7)
c_ELISA	+	-	-	+	66 (37.0)
Total (178)	66 (37.0)	68 (38.2)	44* (24.7)	0 (0.00)	110 (61.7)[#]
	134 (75.2) +		44 (24.7)[$]		

[*Vaccinated cows; n, number; values in parenthesis are percentages. +/- Agreement; [$]disgreement, [#]cumulative bioload of MAP]

Table 4 — Comparative serostatus of JD in known Infected, Negative and Vaccinated cows in i_ELISA *vis-a-vis* c_ELISA test

Serostatus in i_ELISA, *n* (%)		Serostatus in c_ELISA						Status with respect to JD	*n* (%)
		SP	P	LP	S	N			
Positive 110 (61.7)	SP-13	9	8	1	-	-	-	Infected	Infected: 66 (37.0)
	P-97	4	-	-	4	-	-	Vaccinated	
		40	-	-	27	12	1	Vaccinated	Vaccinated: 44 (24.7)
		57	4	53	-	-	-	Infected	
Negative 68 (38.2)	LP-10	-	-	-	10	-			Negative (Healthy)[#]: 68 (38.2)
	S-45	-	-	-	24	21		Negative (Healthy)	
	N-13	-	-	-	2	11			

[*n*, number. Positive (SP, Strong positive; P, Positive); Negative (LP, Low positive; S, Suspected; N, Negative) and [#] Healthy]

cows were in positives (Strong positive 4 and Positive 40) in i_ELISA (Table 4). In cocktail ELISA, all 44 vaccinated cows were partitioned in negative categories (low positive 31, suspected 12, Healthy or non-infected 1) (Table.4). Indigenous Vaccine used 'inactivated MAP' strain 'S 5', therefore whole cell killed MAP strain will not express, secretary antigens. Titers of low positives and suspected cows should have been perfect negative; however, since JD is endemic in domestic livestock population of the country, these low titers were reflection of this fact. Moreover, vaccinated cows shared housing and grazing with other cows with un-known status, which in all probability may not be negative. Due to high bio-load of JD in environment, both vaccinated and non-vaccinated cows were continuously exposed to MAP infection and the residual titers were due to this fact. All 66 cows naturally infected with MAP were in the positive category in both of the ELISA tests (Table.4). However, profiling of cows was better in c_ELISA and of the 66 naturally infected cows, 9 were categories as strong positive (SP) and 57 were in positive (P) category. This is due to the fact that recombinant proteins had better sensitivity and reacted more strongly as compared to Ns_PPA, despite the fact the RSPs were used in very low

quantities. All the 68 apparently healthy cows were detected as negatives in c_ELISA (36 in suspected and 32 in negative categories). These 68 apparently healthy cows were also negative in i_ELISA but were partitioned in low positive (10), suspected (45) and negative (13) categories (Table 4). This is due to the fact that Ns_PPA used in i_ELISA provided a range of proteins from secretory to structural (cytosol) and therefore reacted strongly as compared to c_ELISA where only secretory antigens were used. This also validates the use of both secretory and structural proteins in 'Screening ELISA' test used for primarily screening of livestock. Results showed that c_ELISA if used under the present conditions where disease is highly endemic in cows is able to differentiate infected, vaccinated and healthy animals when used along with i_ELISA. However further validation of the DIVA test using both i_ELISA and c_ELISA in experimentally induced infection in rabbits with known negative, infected and vaccinated status raised on raised platform or on cemented floor with clean forage or grass. On the basis of these results Table 2 has been as Table 5 for the purpose of studies to define the status of cows vaccinated against JD (Table 5).

JD has been reported to be highly endemic in cows herds in a study in India for past >17 years[19] and high bio-load of MAP in domestic livestock is directly correlated with low per animal productivity of the native livestock breeds. At CIRG, Makhdoom for the management and control of JD 'test and cull' method has been employed in goats for past 37 years, without success[19]. After the failure of 'test and cull' strategy (1979 to 2004) at CIRG goat farms, we first time in 2005 developed 'Indigenous Vaccine' using novel bio-type (Indian Bison Type) of goat origin ('strain S 5') of JD[20]. In-house and field trials of 'indigenous vaccine' was showed that both preventive and therapeutic and superior to Gudair vaccine[20]. Since all the domestic livestock (goats, sheep, cows and buffaloes) are infected with 'Indian Bison Type' bio-type[10], the vaccine strain, the indigenous vaccine was equally effective in all the domestic livestock species[10]. However, this 'Indigenous vaccine' cannot be used in the field for the prevention, therapeutic management of JD in domestic livestock unless we develop DIVA test to differentiate between infected and vaccinated cows. Livestock in India lacks identification number, therefore, DIVA test is mandatory before a new vaccine is allowed to be used in the field on mass scale for the control of notifiable diseases like JD.

Secreted proteins have long been acknowledged to play central roles in bacterial-host interactions. Secreted proteins present in the culture filtrate of MAP, have been the focus of this study because MAP specific secretory antigens can also serve as markers for early diagnosis of disease. Comparing the secretory proteins with other cellular proteins showed a greater sero-reactivity in MAP-infected animals. So, secretory proteins were the focus of research for DIVA-based marker assay[21,22].

Some studies suggested that secretory proteins may be better as solid-phase ELISA antigens resulting in a more sensitive assay[23,24]. Pradenas *et al.*[25] reported that most CFPs have low molecular weight and reacted strongly with sera from culture-positive cases of JD. They observed a high degree of difference in CFPs immuno-reactivity in MAP infected animals. Serum samples from cows with clinical JD or heavy faecal shedders of bacilli reacted more intensively to CF proteins. Infected goats and sheep serum samples showed reactivity with CFPs tested in immunoblots. These results suggested that a cocktail of CFPs of MAP could be good candidates as antigens for serodiagnosis of JD. Dheenadhayalan *et al.*[26] also reported immunogenicity of five recombinant antigens, namely MAP2411, ClpP (MAP2281c), Ppa (MAP0435c), 990 MAP0593c and GreA (MAP1027c). 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, MAP0593c 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[27]. Antigens of the Ag85 complex and SOD

Table 5—S/P ratios and 'proposed' corresponding status of non-infected (negative), vaccinated and infected cows for JD / MAP infection using 'c_ELISA'

Calculated value of OD into S/P Ratio	Status of JD in cows*	Status of MAP infection in c_ELISA**
0.00-0.09	Negative (N)	Non-infected (Healthy)
0.10-0.24	Suspected or Borderline (S)	Vaccinated (Suspected)
0.25-0.39	Low Positive (LP)	
0.4-0.99	Positive (P)	Infected
1.0-10.0	Strong Positive (SP)	

[MAP infection S/P ratio categories: N, Negatives; S, Suspected; LP, Low positive; P, Positive; and SP, Strong positive for MAP infection, S+LP, Negative; and P+SP, Positive (Infected). * Adopted from Collin, 2002 in cows[22]; **As per the present study]

showed high reactivity against sera from the MAP shedding cows and little reactivity against sera from the non-shedding cows. Karappusamy *et al*.[28] have identified 15 proteins as product of the genes *sdhA*, *fadE25_2*, *mkl*, *citA*, *gapdh*, *fadE3_2*, *moxR1*, *mmp*, *purC*, *mdh*, *atpG*, *fbpB* and *desA2* in addition to two more proteins identified as transcriptional regulator protein (MAP0035) and hypothetical protein (MAP1233). These proteins are responsible for energy generation, cell wall synthesis, maturation of proteins, replication of bacteria as well as invasion of epithelial cells. These proteins are essential for MAP virulence and survival of bacteria intracellularly. Immunoblot analysis with respective MAP cell envelope proteins showed minimal cross-reactivity with polyclonal antibodies. Developed MAP specific proteins and antibodies may be useful in developing new diagnostic tests for MAP infections[28]. De Souza *et al*.[29] identified the Apa protein which is secreted by MAP as a novel faecal biomarker for diagnosis of MAP infection in cattle.

Conclusion

Present study showed that 'i_ELISA' and 'c_ELISA' using two types antigens; cocktail of six recombinant secretory proteins/antigens (RSPs) and semi-purified Protoplasmic (Ns_PPA) antigens from native MAP biotype (Indian Bison Type) strain S 5, respectively. Results have shown that optimized recombinant secretory proteins based 'Cocktail ELISA' when used along with traditional 'Indigenous ELISA kit' was successful in screening of animals infected with MAP and differentiating infected (diseased), vaccinated and healthy (non-infected) animals. This DIVA assay can facilitate the use of JD vaccine in the control of the disease at natural level.

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Conflict of interest

The authors declare no conflict of Interest.

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