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Pilot scale studies on the optimization of DIVA assay for the inactivated whole cell paratuberculosis vaccine

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ABSTRACT

Paratuberculosis or Johne's disease is chronic enteritis of domestic ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Paratuberculosis results in substantial losses to livestock industry and is also threat to human health, since MAP has zoonotic concerns with Crohn's disease. Therefore, control of this disease is priority to governments. However, widely used 'Test and Cull' control programs have yielded little or no success and alternate approaches are required to control this disease. Vaccination has shown great promise in managing this disease, but there are regulatory restrictions on use of vaccination due to lack of DIVA tool (differentiation of infected and vaccinated animals). In the present study, we report the development of first serology based DIVA tool for the inactivated paratuberculosis vaccine using unique biomarkers present in the secretome of MAP that are not part of vaccine.

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INTRODUCTION

Johne's disease (JD) or Paratuberculosis is a prevalent and costly infectious disease of domestic is a ruminants around the world (Singh *et al.,* 2016). It is chronic granulomatous enteritis and progressive weight loss with or without persistent diarrhea are the clinical signs. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the etiological agent of this disease and is very resistant to environmental stress (like high temperature, drying etc.) and is able to persist for years in farm soil (Singh *et al.,* 2013). Paratuberulosis is endemic in India and disease has been widely reported from domestic livestock population of the country (Singh *et al.,*2014, Sohal *et al.,* 2015). For every clinical case of paratuberculosis, it is likely that 25 more animals are infected on that farm (Whitlock, 1992) and clinical animals are the 'tip of iceberg'. Majority of the animals shed bacilli in their feces without any signs of disease and therefore are continuous source of environmental contamination and infection source for other susceptible animals on that farm.

Paratuberculosis harshly affects the farm economy by reduced milk yield and quality, poor feed conversion, reduced fertility, increased susceptibility to other diseases, premature culling and reduced slaughter value, veterinary expenses etc. MAP is not killed by pasteurization and MAP zoonosis has also become threat as potential food-borne pathogen (Shankar *et al.,* 2010). There are compelling evidences in the involvement of MAP in human Crohn's disease (Singh *et al.,* 2016). Hence paratuberculosis has become notifiable disease and control of this disease has become priorities for the

governments. United States Department of Agriculture (USDA) is implementing Johne's disease control program in USA, similarly, Canadian government has also launched Canadian Johne's Disease Initiative. Australia has also implemented National Johne's Disease Control Program. European Union and Japan have recognized MAP as food borne pathogen and initiated control programs (Kobayashi *et al.,* 2007; Momotani *et al.,* 2012; Nielsen *et al.,* 2009). Immediate objective of these programs is to prevent the spread of infection and eradication is the long term goal. 'Test and Cull' policy with management changes is the backbone of these control programs (Sohal *et al.,* 2015). Some nations like Germany, Denmark, Ireland, Czech Republic, Austria, Norway etc. also compensate their farmers for culling the paratuberculosis infected animals (Khol and Baumgartner, 2014; Kralik and Slana, 2014; Amely *et al.,* 2014, Østerås, 2014). To our knowledge no developing country has initiated paratuberculosis control program besides notable prevalence in countries like India, Mexico, Brazil, China etc. (Milián-Suazo *et al.,* 2015; Singh *et al.,* 2014; Liu *et al.,* 2017; Vilar *et al.,* 2015). Major reason for developing countries for not adopting the control program is the costly nature of the test and cull policy and governments in developing countries cannot afford compensation for culled animals. Moreover, in India due to religious sentiments cattle slaughter in banded in majority of the provinces. Recently findings of test and cull policy has been reviewed and it has been analyzed that test and culling to date has not produced the expected results (Bastida and Juste, 2011) due to lack of procedures to diagnose disease in early stages (Jayaraman *et al.,* 2016). Therefore, in order to improve the efficacy of present control programs in developed countries and popularize the control of paratuberculosis in developing world alternate low cost control strategies are needed to be developed.

Experiences from past and recent findings suggest that vaccination against paratuberculosis is the most practical method to control paratuberculosis (Jayaraman *et al.,* 2016). Compared to test and cull based control methods, studies have established vaccination as the economic method to control paratuberculosis (Dhand *et al.,* 2013; Juste and Casal, 1993; van Schaik *et al.,* 1996).Vaccination offers various advantages, like reduces morbidity & mortality due to paratuberculosis, reduces shedding of MAP in feces, improves clinical condition (reduces diarrhea & increases body weight), cures intestinal lesions and enhances flock immunity to JD (Singh *et al.,* 2007; Singh *et al.,* 2010b; Singh *et al.,* 2013). Studies have confirmed that vaccination not only reduces the prevalence of JD but also brings economic benefits to farmers (Groenendaal *et al.,* 2015). The most significant advantage of paratuberculosis vaccination is therapeutic effects (Singh *et al.,* 2010b). This is very substantial to Indian context since infected cattle cannot be culled, so vaccination is the best solution to manage the infected cattle. Killed whole cell vaccines are preferred over other types due to their safety and higher stability.

Although vaccination for paratuberculosis has many advantages, but there are regulatory restrictions on mass and field use of paratuberculosis vaccine (Patton, 2011). Reason of regulatory restrictions is our inability to discriminate between infected and vaccinated animals within the herd, because paratuberculosis bacilli shares antigenic structures with *M. bovis* (responsible for bovine tuberculosis). Vaccination for paratuberculosis will therefore interfere with serology and DTH based diagnosis of both diseases. Hence DIVA tool is required for the field and free use of paratuberculosis vaccine. In the present study, we have optimized DIVA tool in plate ELISA format for whole cell killed paratuberculosis vaccine using secretory proteins. Since secretory proteins are not part of vaccine, so antibodies to these proteins should be absent in vaccinated individuals. The detailed strategy has been explained by Jayaraman *et al.,* (2016).

MATERIALS AND METHODS

Selection of the secretory proteins, cloning and expression

Four secretory proteins (MAP1693c, MAP2677c, MAP3547c and MAP4308c) were selected. These proteins have been reported to be immuno-dominant in previous studies (Roupie *et al.,* 2008; Mon *et al.,* 2012; Roupie *et al.,* 2012). Nucleotide sequences of these proteins were subjected to codon optimization using GeneOptimizer software. Optimized gene sequences were synthesized commercially from Thermo Fisher Scientific, USA and cloned in pET151/D-TOPO expression vector.

Recombinant plasmid was transformed to *E. coli* BL-21 cells for expression as per Sohal *et al.,* 2008. Expression was done in 1000 ml LB broth containing ampicillin (100µg/mL) using IPTG stimulation at final concentration of 0.3 mM, after stimulation cells were incubated for 24 hours at 37°C, cells were pelleted and suspended in 5 ml equilibration buffer (50 mM, TrisHCl, 200 mM NaCl, 5mM DTT, 1mM PMSF, pH 8.0). The suspension was subjected to sonication (20 cycles of 1 minute with 1 minute interval at 45% amplitude). Recombinant proteins were further purified by nickel-nitrilotriacetic acid (Ni-NTA) gel matrix. Purified protein was analyzed by SDS-PAGE (Gupta *et al.,* 2015) and concentration was taken using Nanodrop. Immunogenicity of

Table 1: Analysis of the serum samples of vaccinated cattle using PPD ELISA and DIVA ELISA

S. No.	Animal ID	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
1	36A	0.57	0.27
2	15c	0.82	0.21
3	33b	0.47	0.27
4	17b.2	0.75	0.33
5	22A	0.66	0.39
6	1	0.73	0.10
7	13A	0.68	0.16
8	129	0.43	0.24
9	2	0.71	0.23
10	121	0.68	0.39
11	19	0.60	0.18
12	168(m)	0.42	0.31
13	128	0.53	0.14
14	tag63	0.41	0.17
15	tag226f	0.47	0.17
16	tag53	0.82	0.18
17	tag37	0.46	0.10
18	tag46	0.80	0.20
19	47	0.52	0.31
20	13a	0.49	0.28

Table 2: Analysis of the serum samples of naturally infected cattle using PPD ELISA and DIVA ELISA

S. No.	Animal ID	PPD ELISAS/P ratio	DIVA ELISAS/P ratio
1	8a	0.52	0.72
2	3	0.53	0.81
3	19a	0.48	0.86
4	23a	0.72	1.10
5	9	1.18	1.72
6	12	0.58	0.93
7	32b	0.62	0.66
8	17b	0.53	1.12
9	23b	0.78	0.81
10	35b	0.44	1.23
11	20 (red)	0.78	0.46
12	124	0.72	0.45
13	19	0.93	0.46
14	22M buff	0.91	0.43
15	12a	0.52	0.51
16	tag233f	0.50	0.71
17	tag07	0.60	1.04
18	18	0.84	0.42
19	11	0.78	0.54
20	bull3	0. 90	1.00

the proteins was confirmed by standard immunoproteomic analysis (Gupta *et al.,* 2015) using positive control sera from cattle, goat, sheep and buffalo.

Optimization of DIVA ELISA

An indirect plate ELISA was optimized to differentiate vaccinated and naturally infected animals using cocktail of the above four recombinant proteins using traditional checker board titration technique as per Singh et al. (2011). ELISA OD values were converted into S/P ratio for presentation of results. Sera of vaccinated, infected and healthy animals used for optimization of the assay were provided by Central Institute for Research on Goats (CIRG), Mathura (Table 1-3). The serum samples were also analyzed by traditional ELISA based on PPD developed by CIRG, Mathura. After this initial optimization, DIVA ELISA was evaluated on the extended panel of sera from field samples available with the repository of CIRG, Mathura. In total 104 sera from

Table 3: Analysis of the serum samples of healthy cattle using PPD ELISA and DIVA ELISA

S. No.	Animal ID	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
1	5	0.04	0.01
2	3(M)	0.05	0.01
3	223(f)	0.09	0.03
4	tag104	0.09	0.05
5	3	0.06	0.04
6	pb14	0.02	0.04
7	pb6	0.04	0.04
8	pb1	0.01	0.07
9	2C	0.02	0.06
10	CN	0.01	0.08

Table 4: Analysis of the extended panel of serum samples from vaccinated animals of different species using PPD ELISA and DIVA ELISA

S. No.	Animal ID	Farm/ Herd	Specie	Months Post Vaccination	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
1	JPF-1	J.P. Farm, Etawah, UP	Goat	12	0.53	0.26
2	JPF-2				0.47	0.24
3	JPF-3				0.89	0.39
4	JPF-4				0.83	0.36
5	JPF-5				0.57	0.28
6	JPF-6				0.78	0.32
7	JPF-7				0.85	0.40
8	JPF-8				0.80	0.40
9	JPF-9				0.67	0.25
10	JPF-10				0.71	0.29
11	EGF_G-1	E.G.L. Farm, Sundrel, MP	Goat	18	1.04	0.40
12	EGF_G -2				1.17	0.40
13	EGF_G -3				0.74	0.31
14	EGF_G -4				0.99	0.39
15	EGF_G -5				0.69	0.28
16	EGF_G -6				1.01	0.37
17	EGF_G -7				0.62	0.19
18	EGF_G -8				0.49	0.21
19	EGF_G -9				0.55	0.23
20	EGF_G -10				0.61	0.19
21	EGF_G -11				0.58	0.21
22	EGF_G -12				0.97	0.28
23	EGF_G -13				0.84	0.27
24	EGF_G -14				0.91	0.40
25	EGF_G -15				0.72	0.26
26	UGF-1	U.G. Farm, Garh-Mukteshwar, UP	Goat	09	0.83	0.37
27	UGF-2				0.72	0.36
28	UGF-3				0.65	0.28
29	UGF-4				0.73	0.25
30	UGF-5				0.92	0.40
31	BGF-1	B.G.Farm, Gurgaon, Haryana	Goat	12	0.89	0.33
32	BGF-2				0.77	0.40
33	BGF-3				0.72	0.27
34	BGF-4				0.69	0.21
35	BGF-5				0.66	0.19
36	BGF-6				0.96	0.35

Table 4: Analysis of the extended panel of serum samples from vaccinated animals of different species using PPD ELISA and DIVA ELISA (Continued…)

37	KGF-1	K.G. Farm, Mathura, UP	Goat	15	0.78	0.23
38	KGF-2				0.80	0.30
39	KGF-3				0.85	0.26
40	KGF-4				0.87	0.27
41	KGF-5				0.76	0.31
42	KGF-6				0.58	0.39
43	KGF-7				0.63	0.28
44	KGF-8				0.61	0.31
45	KGF-9				0.57	0.40
46	KGF-10				0.92	0.32
47	KGF-11				1.04	0.38
48	KGF-12				0.87	0.21
49	KGF-13				0.78	0.28
50	KGF-14				0.56	0.35
51	KGF-15				0.62	0.22
52	KGF-16				0.73	0.37
53	KGF-17				0.81	0.21
54	KGF-18				0.63	0.33
55	KGF-19				0.77	0.37
56	KGF-20				0.83	0.28
57	BDF-1	B.D. Farm, Gurgaon, Haryana	Cattle	12	0.80	0.30
58	BDF-2				0.82	0.22
59	BDF-3				0.76	0.36
60	BDF-4				0.68	0.28
61	BDF-5				0.72	0.37
62	BDF-6				0.66	0.26
63	BDF-7				0.97	0.27
64	BDF-8				0.94	0.36
65	BDF-9				0.83	0.19
66	BDF-10				0.72	0.21
67	KD_M-1	K.D.Farm, Mathura, UP	Cattle	18	0.89	0.40
68	KD_M -2				0.69	0.28
69	KD_M -3				0.96	0.35
70	KD_M -4				0.78	0.32
71	KD_M -5				0.80	0.20
72	KD_M -6				0.85	0.35
73	KD_A-1	K.D. Farm, Agra, UP	Cattle	18	0.58	0.28
74	KD_A-2				0.67	0.36
75	KD_A-3				0.68	0.38
76	KD_A-4				0.72	0.22
77	EGL_C-1	E.G.L.Farm, Sundrel, MP	Cattle	06	0.71	0.32
78	EGL_C-2				0.61	0.33
79	DDK-1	D.D.K. Gausala, Farah, UP	Cattle	18	0.75	0.35
80	DDK-2				0.62	0.26
81	DDK-3				0.56	0.35
82	DDK-4				0.83	0.23
83	DDK-5				0.59	0.39
84	DDK-6				0.92	0.28
85	DDK-7				0.88	0.39
86	DDK-8				0.73	0.34

Table 4: Analysis of the extended panel of serum samples from vaccinated animals of different species using PPD ELISA and DIVA ELISA (Continued…)

87	GGF-1	G.G. Farm, Ma-	Cattle	24	0.77	0.29
88	GGF-2	khdoom, UP			0.55	0.28
89	GGF-3				0.61	0.36
90	GGF-4				0.99	0.38
91	GGF-5				1.01	0.40
92	GGF-6				0.58	0.28
93	GGF-7				0.64	0.31
94	GGF-8				0.91	0.36
95	GGF-9				0.88	0.38
96	GGF-10				0.77	0.31
97	KBF-1	K.B. Farm, Mathura,	Buffalo	18	0.69	0.25
98	KBF-2	UP			0.99	0.34
99	KBF-3				0.76	0.29
100	KBF-4				0.58	0.21
101	KBF-5				0.71	0.29
102	KBF-6				0.82	0.35
103	KBF-7				0.89	0.39
104	KBF-8				1.01	0.39

Table 5: Analysis of the extended panel of serum samples from naturally infected animals of different species using PPD ELISA and DIVA ELISA

S.No.	Animal ID	Species	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
1	37A	Goat	0.57	0.77
2	33	Cattle	0.56	0.59
3	23	Cattle	0.93	0.74
4	32A	Goat	0.88	0.66
5	40	Cattle	0.85	0.42
6	47	Cattle	0.84	0.76
7	34 New	Cattle	1.00	0.58
8	20	Cattle	1.18	0.60
9	39	Cattle	0.76	0.58
10	35	Cattle	0.98	0.71
11	10	Cattle	1.43	1.61
12	17b	Buffalo	1.0000	0.71
13	35 old	Cattle	0.79	0.50
14	16	Cattle	0.90	0.70
15	18	Cattle	0.75	0.51
16	28a	Goat	1.03	0.55
17	3	Cattle	0.72	0.84
18	40a	Goat	0.99	0.62
19	30	Cattle	1.27	0.53
20	21	Sheep	0.79	0.68
21	22	Sheep	1.071	0.78
22	3	Sheep	0.73	0.75
23	39	Sheep	0.93	0.49
24	35	Sheep	0.74	0.64
25	33A	Goat	0.87	0.68
26	32B	Buffalo	0.76	0.62
27	18A	Goat	0.50	0.54
28	16A	Goat	0.62	0.79
29	28	Sheep	0.64	0.53
30	17b.2	Buffalo	0.75	0.53

Table 5: Analysis of the extended panel of serum samples from naturally infected animals of different species using PPD ELISA and DIVA ELISA (Continued…)

S.No.	Animal ID	Species	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
31	22A	Goat	0.66	0.79
32	10A	Goat	0.59	0.51
33	5	Cattle	0.55	0.77
34	11	Cattle	0.63	0.85
35	35	Cattle	0.70	0.67
36	60	Cattle	0.64	0.73
37	8a	Goat	0.52	0.72
38	3	Cattle	0.53	0.81
39	19a	Goat	0.58	0.86
40	47a	Goat	0.57	0.92
41	23a	Goat	0.72	1.10
42	9	Cattle	1.18	1.72
43	12	Cattle	0.58	0.93
44	17b	Buffalo	0.53	1.12
45	13a	Goat	0.67	0.56
46	27a	Goat	0.61	0.94
47	23c	Cattle	0.58	0.52
48	51	Sheep	0.54	0.74
49	10	Sheep	0.66	0.96
50	28a	Goat	0.95	0.65
51	23b	Buffalo	0.77	0.81
52	35b	Buffalo	0.54	1.23
53	36a	Goat	0.52	0.54
54	6b	Buffalo	0.71	0.77
55	4	Cattle	0.60	0.57
56	35a	Goat	0.66	0.60
57	25a	Goat	0.88	0.75
58	52	Cattle	0.51	0.78
59	33b	Buffalo	1.21	0.71
60	52 buff	Buffalo	0.61	0.60
61	227	Cattle	0.80	0.68
62	26	Cattle	0.74	0.55
63	117	Cattle	0.63	0.50
64	cow 0029	Cattle	0.69	0.67
65	cow 0014	Cattle	1.39	1.46
66	9	Cattle	0.59	0.77
67	34	Cattle	0.54	0.48
68	1	Cattle	0.57	0.71
69	N.06	Cattle	1.08	1.18
70	N.07	Cattle	0.72	0.68
71	N.08	Cattle	0.42	0.68
72	N.10	Cattle	0.63	0.95
73	143	Cattle	0.56	0.58
74	tag19	Cattle	0.75	0.88
75	tag206f	Cattle	0.41	0.64
76	tag75	Cattle	0.55	0.65
77	tag43	Cattle	0.52	0.63
78	tag48	Cattle	0.60	0.65
79	tag75	Cattle	0.67	0.95
80	tag07	Cattle	0.60	1.04

Table 5: Analysis of the extended panel of serum samples from naturally infected animals of different species using PPD ELISA and DIVA ELISA (Continued…)

S.No.	Animal ID	Species	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
81	tag31	Cattle	0.53	0.75
82	bull2 cn	Buffalo	0.50	0.73
83	40	Cattle	0.73	0.52
84	6	Cattle	1.42	0.63
85	25	Cattle	0.73	0.53
86	12	Cattle	1.09	1.29
87	3	Cattle	0.68	0.43
88	13	Cattle	0.72	0.53
89	bull2 cn	Buffalo	0.52	1.18
90	7	Cattle	1.46	0.64

Table 6: Analysis of the extended panel of serum samples from healthy animals of different species using PPD ELISA and DIVA ELISA

S.No.	Animal ID	Species	PPD ELISA S/P ratio	DIVA ELISA S/P ratio
1	26	Goat	0.06	0.08
2	3	Goat	0.01	0.01
3	47	Goat	0.09	0.02
4	30	Goat	0.05	0.07
5	13a	Sheep	0.01	0.03
6	5	Goat	0.04	0.01
7	12a	Sheep	0.02	0.01
8	3(M)	Sheep	0.05	0.01
9	3	Goat	0.05	0.04
10	pc2	Cattle	0.04	0.08
11	pc12	Cattle	0.05	0.06
12	pb13	Cattle	0.09	0.01
13	pb1	Cattle	0.01	0.07
14	tag104	Cattle	0.03	0.05
15	pb14	Cattle	0.01	0.01
16	pb6	Cattle	0.03	0.03
17	223(f)	Buffalo	0.07	0.03

vaccinated animals, 90 sera from naturally infected animals and 17 sera from healthy animals were used to validate the DIVA ELISA (Table 4-6). These sera were also analyzed by PPD based ELISA developed by CIRG, Mathura.

RESULT AND DISCUSSION

Immuno-proteomic analysis confirmed that selected antigens are strong immunogens for domestic ruminant species (cattle, goat, sheep and buffalo) (Fig. 1-4). Checker board titration results showed that 10 ng per well of each antigen is optimal for the coating the wells of the ELISA plate. DIVA ELISA optimization results showed that based on the S/P ratio we can distinguish vaccinated, infected and healthy animals with respect to paratuberculosis (Table 1-3). Healthy animals had S/P ratio in the ≤0.09, vaccinated animals had S/P ratio ≤0.40 and infected animals had S/P ratio >0.40. Validation of DIVA ELISA on extended panel of the sera also confirmed the optimization findings (Table 4-6). It was found that PPD based ELISA is unable to distinguish vaccinated and naturally infected animals (Table 1-6).

Global burden of paratuberculosis is mounting (Singh *et al.,* 2014) and due to failure of the 'Test and Cull' programs, there is need to develop alternate disease control strategies. Vaccination has been recognized as better alternate to 'Test and Cull', however, vaccination will interfere with the routine diagnosis of disease and will be problem in herd certification programs, therefore for the field use of vaccine, DIVA tool is needed. DIVA tool will not only popularize the paratuberculosis vaccination but will also boost the ongoing programs. Concept of DIVA has been well-established for veterinary vaccines and DIVA technology has been recognized as unprecedented achievement in veterinary world for its ethical, environmental, and social-economic benefits towards animal and human well-being (Domínguez *et al.,* 2014).

In the present study, we report the development of first DIVA assay for inactivated paratuberculosis

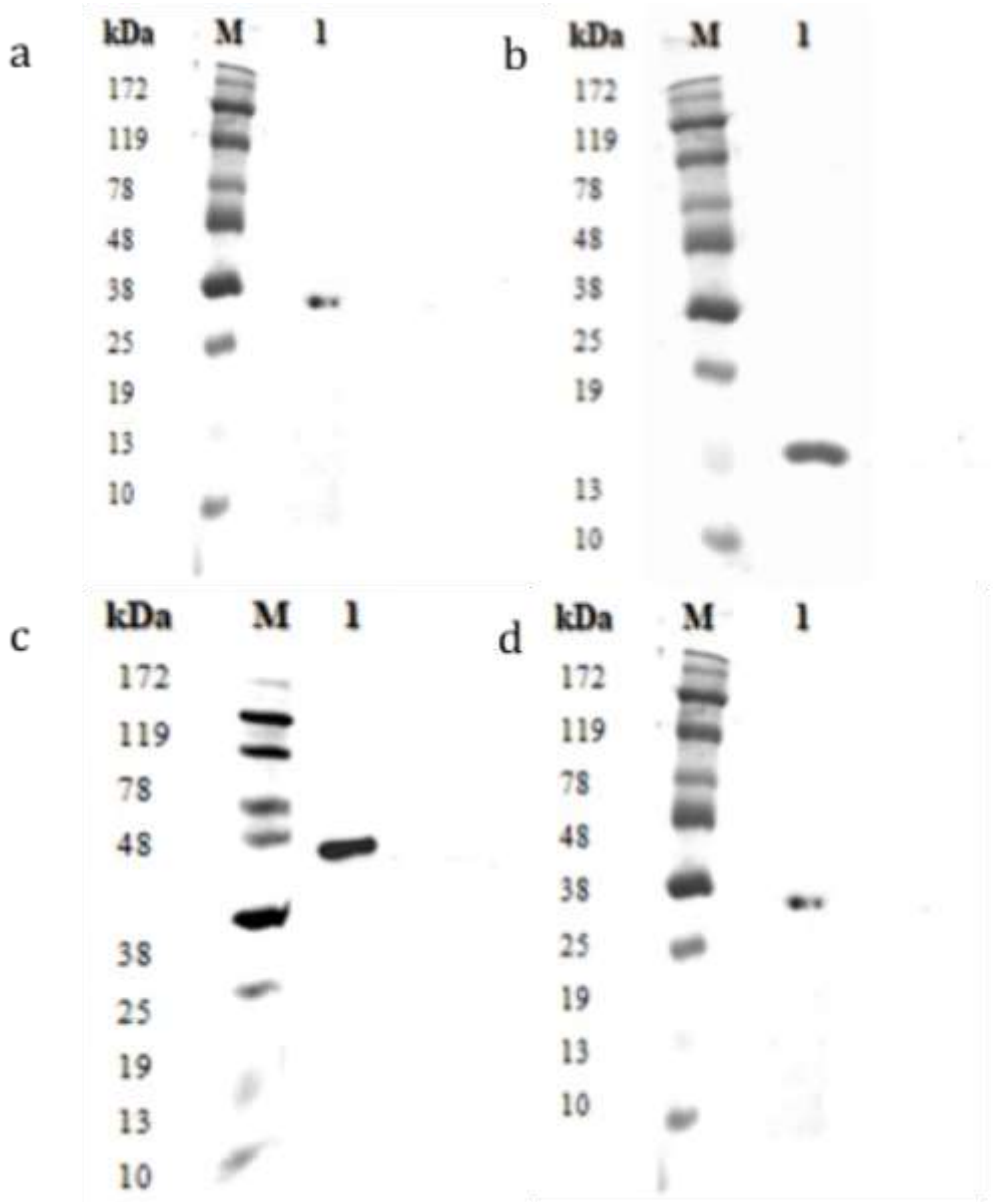

Figure 1: Immuno-proteiomic analysis of recombinant proteins using goat serum a) Left to right; M: pre-stained protein ladder, Lane 1: MAP1693c; b) Left to right; M: pre-stained protein ladder, Lane 1: MAP2677c; c) Left to right; M: pre-stained protein ladder, Lane 1: MAP4308c; d) Left to right; M: pre-stained protein ladder, Lane 1: MAP3547c

vaccine. Like the majority of available DIVA tools (Domínguez *et al.,* 2014), presently study also exploited the immunogens that are active during the natural infection and are not part of vaccine. We used secretory antigenic biomarkers (MAP1693c, MAP2677c, MAP3547c and MAP4308c) to develop DIVA. These secretory antigens are specific to MAP and are absent from *M. tuberculosis* complex (Leroy *et al.,* 2007; Mikkelsen *et al.,* 2012). These proteins are secreted during the active growth of mycobacteria in the host (Waghmare *et al.,* 2016) and strong humoral and cell mediated responses have been reported against secretory proteins of mycobacteria during natural infection (Wu *et al.,* 2010, Xu *et al.,*2008). So, serological response against these antigens should be present in naturally infected animals but as these antigens are not part of vaccine so antibodies to these proteins should be absent in vaccinated individuals. Immuno-dominance of these proteins to all domestic ruminant species (cattle, goat, sheep and buffalo) was first time confirmed in this studies, previous studies only analyzed either in cattle or sheep (Leroy *et al.,* 2007; Mikkelsen *et al.,* 2012).

Optimized DIVA ELISA showed promise and could differentiate vaccinated and infected animals. Since ELISA OD values may vary with kind of reagents used or person to person handling variations. So, a unique standard was needed to predict the results of the ELISA, in the present study we used the S/P ratio criterion (Collins *et al.,* 2002) to

analyze the findings of the DIVA ELISA. We found a consistent S/P ratio range for vaccinated (<0.40), naturally infected (>0.40) and healthy animals (≤0.09) with respected to paratuberculosis both in our optimization studies as well as laboratory scale validation studies. Serology based DIVA tool has successfully been developed for other veterinary diseases viz. rinderpest virus (RPV), peste des petits ruminants viruses (PPRV), bovine herpesvirus 1 (BHV-1), Aujeszky's diseases, foot & mouth disease virus (FMDV) and *Salmonella* (Vordermeier *et al.,* 2016; Bearson *et al.,* 2016; Domínguez *et al.,* 2014; Lee *et al.,* 2012). DIVA strategy has been applied successfully for the control of pseudorabies in pigs and avian influenza (Pasick, 2004). In conclusion, results of the present study are promising and DIVA ELISA has the potential to be used in field with vaccination and field validation of the DIVA ELISA is need for more stringent confirmation.

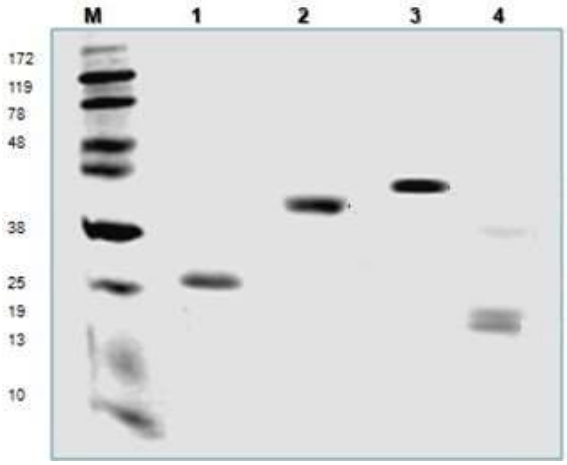

Figure 2: Immuno-proteiomic analysis of recombinant proteins using cattle serum, left to right; M: pre-stained protein ladder, Lane 1: MAP 1693c, Lane 2: MAP 4308c, Lane 3: MAP 3547c, Lane 4: MAP 2677c

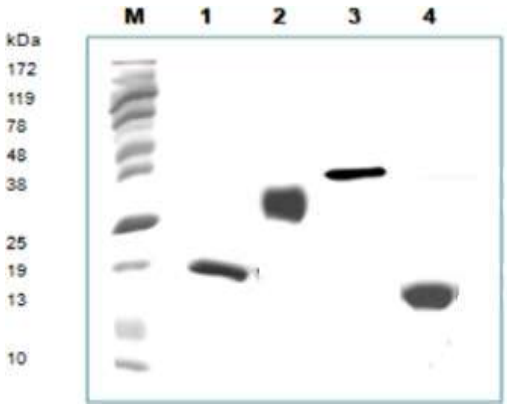

Figure 3: Immuno-proteiomic analysis of recombinant proteins using buffalo serum, left to right; M: pre-stained protein ladder, Lane 1: MAP1693c, Lane 2: MAP4308c, Lane 3: MAP3547c, Lane 4: MAP2677c

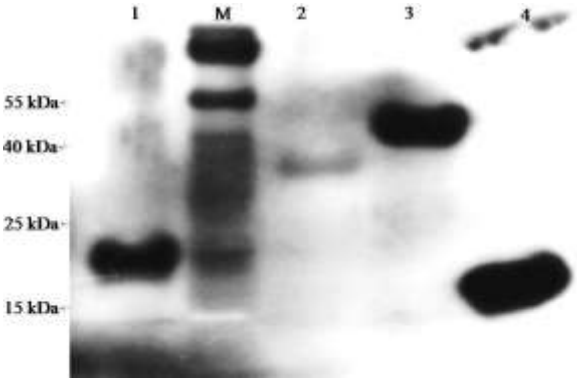

Figure 4: Immuno-proteiomic analysis of recombinant proteins using sheep serum, left to right; Lane 1: MAP1693c, M: pre-stained protein ladder, Lane 2: MAP4308c, Lane 3: MAP3547c, Lane 4: MAP2677c

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REFERENCES

Bastida, F., & Juste, R. A. (2011). Paratuberculosis control: a review with a focus on vaccination. *Journal of Immune Based Therapies and Vaccines*, 9: 8.

Bearson, B.L., Bearson, S.M.D., Brunelle, B.W., Bayles, D.O., Lee, I.S., Kich, J.D., (2016). Salmonella DIVA vaccine reduces disease, colonization and shedding due to virulent *S. Typhimurium* infection in swine. *Journal of Medical Microbiology,* 66(5), 651-661.

Collins, M. T. (2002). Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using likelihood ratios. *Clinical and Diagnostic Laboratory Immunology*, *9*(6), 1367-1371.

Dhand, N. K., Johnson, W. O., Eppleston, J., Whittington, R. J. & Windsor, P. A. (2013). Comparison of pre- and post-vaccination ovine Johne's disease prevalence using a Bayesian approach. *Preventive Veterinary Medicine,* 111, 81-91.

Dominguez, M., Gache, K., Touratier, A., Perrin, J.B., Fediaevsky, A., Collin, E., Breard, E., Sailleau, C., Viarouge, C., Zanella, G., Zientara, S., Hendrikx, P., Calavas, D. (2014). Spread and impact of the Schmallenberg virus epidemic in France in 2012-2013. BMC *Veterinary Research*, 10, 248.

Groenendaal H., Zagmutt, F. J., Patton, E. A. & Wells, S. J. (2015). Costbenefit analysis of vaccination

against Mycobacterium avium ssp. paratuberculosis in dairy cattle, given its cross-reactivity with tuberculosis tests. *Journal of Dairy Science*, 98, 6070-84.

Gupta, S., Chaubey, K.K., Singh, S.V., Bhatia, A.K., Kumar ,N., Goel, A., Sachan, T.K., Rawat, K.D., Sohal J.S. & Dhama, K. (2015). Immunoreactivity to culture filtrate proteins of *Mycobacterium avium* subspecies *Paratuberculosis* in naturally infected goat and sheep sera. *Advances in Animal and Veterinary Sciences,* 3, 347-353.

Jayaraman S., Mukta Jain, Kuldeep Dhama, S. V. Singh, Manali Datta, Neelam Jain, K.K. Chaubey, S. Gupta, G. K. Aseri, Neeraj Khare, Parul Yadav, A. K. Bhatia, J. S. Sohal. DIVA Technology: Indispensable Tool for the Control of Johne's disease. Journal of Experimental Biology and Agricultural Sciences, 2016, 4 (16-25)

Juste RA & Casal J. An economic and epidemiologic simulation of different control strategies for ovine paratuberculosis. Prev Vet Med. 1993;15:101–115. doi: 10.1016/0167-5877(93)90106-4.

Khol, J.L. & Baumgartner, W. (2012). Examples and suggestions for the control of paratuberculosis in European cattle. Jpn J Vet Res. Suppl: S1-7.

Kobayashi S., Tsutsui T., Yamamoto T., Nishiguchi A. (2007). Current surveillance and control program on paratuberculosis in Japan. *Bull. Int. Dairy Fed.* 410:30.

Kralik P, Pribylova-Dziedzinska R, Kralova A, Kovarcik K, Slana I. (2014). Evidence of passive faecal shedding of Mycobacterium avium subsp. paratuberculosis in a Limousin cattle herd. Veterinary Journal. 201(1): 91-4.

Lee, N. H., Lee, J. A., Park, S. Y., Song, C. S., Choi, I. S. & Lee, J. B. (2012). A review of vaccine development and research for industry animals in Korea. *Clinical and experimental vaccine research*, *1*(1), 18-34.

Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K. & Wattiez, R. (2007). Antigen discovery: a post-genomic approach to paratuberculosis diagnosis. *Proteomics*, 7, 1164-1176.

Momotani, E., Ozaki, H., Hori, M., Yamamoto, S., Kuribayashi, T., Eda, S., & Ikegami, M. (2012). *Mycobacterium avium* subsp. *paratuberculosis* lipophilic antigen causes Crohn's disease-type necrotizing colitis in Mice. *SpringerPlus*, *1*(1), 47.

Mon, M.L., Viale, M., Baschetti, G., Alvarado Pinedo, F., Gioffre, A., Travería, G., Willemsen, P., Bakker, D., Romano, M.I. (2012). Search for *Mycobacterium avium* subspecies *paratuberculosis* antigens for the diagnosis of paratuberculosis. *Veterinary Medicine International*, 2012, 860362. doi: 10.1155/2012/860362.

Nielsen SS, Krogh MA, Enevoldsen C (2009). Time to the occurrence of a decline in milk production in cows with various paratuberculosis antibody profiles. *Journal of Dairy Science*, 92: 149-55.

Pasick, J. (2004). Application of DIVA vaccines and their companion diagnostic tests to foreign animal disease eradication. Animal Health Research Reviews, 5, 257–262.

Patton EA. Paratuberculosis vaccination (2011). Veterinary Clinics of North America: Food Animal Practice, 27(3): 573-80.

Roupie, V., Leroy, B., Rosseels, V., Piersoel, V., Noël-Georis, I., Romano, M. & Huygen, K. (2008). Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of Mycobacterium avium subsp. paratuberculosis secretome. *Vaccine*, *26*(37), 4783-4794.

Roupie, V., Viart, S., Leroy, B., Romano, M., Trinchero, N., Govaerts, M. & Huygen, K. (2012). Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and Map infected mice. *Veterinary immunology and immunopathology*, *145*(1), 74-85.

Shankar, H., Singh, S. V., Singh, P. K., Singh, A. V., Sohal, J. S. & Greenstein, R. J. (2010). Presence, characterization, and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk, and milk products in India by culture, PCR, and PCR-REA methods. *International Journal of Infectious Diseases*, *14*(2), e121-e126.

Singh, A. V., Chauhan, D. S., Singh, S. V., Kumar, V., Singh, A., Yadav, A., & Yadav, V. S. (2016). Current status of *Mycobacterium avium* subspecies *paratuberculosis* infection in animals & humans in India: What needs to be done? *The Indian Journal of Medical Research*, *144*(5), 661–671

Singh, A.V., Singh, S.V., Verma, D.K., Yadav, R., Singh, P.K. & Sohal, J.S. (2011). Evaluation of "Indigenous Absorbed ELISA Kit" for the Estimation of Sero-prevalence of Mycobacterium avium subspecies paratuberculosis Antibodies in Human Beings in North India. Veterinary Sciences, doi: 10.5402/2011/636038.

Singh, S. V., Singh, M. K., Singh, N. P., Singh, P. K., Singh, A. V., Sohal, J. S., Gupta, V. K. & Vihan, V. S. (2007). Quick and early response to vaccination and improvements in production traits in naturally infected breeding farm of Jamunapari goats in India using indigenous vaccine against Johne's

disease. In: Neilsen, S. S., (Ed). Proceedings of *Ninth International Colloquium on Paratuberculosis, Tsukuba, Japan*, p-225.

Singh, S. V., Singh, P. K., Singh, A. V., Sohal, J. S. & Kumar, N. (2014). Bio-load and bio-type profiles of *Mycobacterium avium* subspecies *paratuberculosis* infection in the domestic livestock population endemic for Johne's disease: a survey of 28 years (1985-2013) in India, *Transboundary Emerging Diseases,* 61, 43-55.

Singh, S. V., Singh, P. K., Singh, A. V., Sohal, J. S. & Sharma, M. C. (2010b). 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 Goatherds in India. *Veterinary Medicine International*, 2010, 351846.

Singh, S.V., Kumar, N., Singh, S.N., Bhattacharya, T., Sohal, J.S., Singh P.K., Singh, A.V., Singh, B., Chaubey, K.K., Gupta, S., Sharma, N., Kumar, S. & Raghava G.P.S. (2013b) Genome sequence of the "Indian Bison type" biotype of *Mycobacterium avium* subsp. *paratuberculosis* strain S 5. *Journal of Bacteriology: Genome Announcement,* 1(1), e00005-13.

Singh, S.V., Stephen, B.J., Singh, M., Gupta, S., Chaubey, K.K., Sahzad, Jayaraman, S., Aseri, G.K., Sohal, J.S., Bhatia, A.K., Chauhan, J. & Dhama, K. (2016c). Evaluation of milk dot-ELISA as field based test vis a vis milk plate ELISA for the detection of Mycobacterium avium subspecies paratuberculosis (MAP) in lactating domestic livestock. Indian Journal of Biotechnology, 15, 166-171.

Sohal J.S., Singh S.V., Singh B., Thakur S., Aseri G.K., Jain N., Jayaraman S., Yadav P., Khare N., Gupta S., Chaubey K.K., Dhama K. (2015). Control of paratuberculosis: opinions and practices. *Advances in Animal and Veterinary Sciences.* 3(3): 156-163.

Sohal, J. S., Singh, S. V., Tyagi, P., Subhodh, S., Singh, P. K., Singh, A. V., Narayanasamy, K., Sheoran, N., Singh, R. & Sandhu, K. (2008). Immunology of mycobacterial infections: with special reference to Mycobacterium avium subspecies paratuberculosis. Immunobiology, 213, 585-98.

Van Schaik G, Kalis CH, Benedictus G, Dijkhuizen AA, Huirne RB. Cost-benefit analysis of vaccination against paratuberculosis in dairy cattle. Vet Rec. 1996;139:624–627.

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G. (2001). Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in Cattle. *Clinical and Diagnostic Laboratory Immunology,* 8, 571–578.