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Recombinant Proteins based 'rELISA' Field Validation for Differential Diagnosis of Johne's Disease in Goats

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ABSTRACT

Background: Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is spreaded worldwide in domestic livestock. It is very clear that JD control program having tedious complications so, only 'Test and cull' and 'vaccination' strategies are key components in JD control program. 'Test and cull' policy is not feasible in Indian condition due to religious beliefs However, vaccination interferes with the immunological tests used for screening of infected and vaccinated (Healthy) animals. Thus, a test that can differentiate between infected and vaccinated animals (DIVA) is needed before implementation of vaccination program.

Methods: Herein, we evaluated two in-house ELISA tests *i.e.,* recombinant proteins-based ELISA (rELISA); developed by using six MAP specific recombinant culture filtrate proteins and indigenous ELISA (i_ELISA); developed using whole-cell protoplasmic MAP antigens for their potential. Sera collected from goats under 'infected', 'healthy and vaccinated' and 'healthy and non-vaccinated' groups were tested by both the tests.

Result: On analyzing the anti-MAP antibodies level detected by both the ELISAs and anti-MAP antibodies against secretary proteins only detected in i_ELISA, it was concluded that rELISA can differentiate healthy, vaccinated and infected goats, if used in-combination with i_ELISA. Therefore, recombinant proteins based rELISA has potential to be considered as companion tool for future diagnostic for successful implementation of JD vaccination control program.

Key words: Culture filtrate protein, Indigenous ELISA, Johne's disease, *Mycobacterium avium* subspecies *paratuberculosis,* Recombinant proteins-based ELISA.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) responsible to cause Johne's disease (JD) in a domestic ruminants, is a chronic enteritis leading to high indisposition, economic and production losses. Complexities caused due to JD may invite trade restrictions at both the international and national levels (Kennedy *et al.,* 2017; Chaubey *et al.,* 2017). Infected animals shed MAP bacilli into milk and thus, can transmit to other animals and humans. Substantial evidence is now available which suggesting that MAP may cause Crohn's disease in humans (Singh *et al.,* 2014a). Among different biotypes of MAP, 'Indian-Bison Type' is the most predominant biotype in India; and it has been detected in broad ruminants species (wild and domestic), other animals like rabbits, primates and human beings (Singh *et al.,* 2014a; 2014b; Chaubey *et al.,* 2016; 2017; Biswal *et al*., 2020; Audarya *et al*., 2022). Based on multiple tests (ZN-staining, i_ELISA, culture and IS*900* PCR *etc*.), the prevalence of MAP is reported to be 20.0, 10.6, 26.6 and 35.1% in cattle, buffaloes, goats and sheep, respectively (Chaubey *et al.,* 2017).

JD control program is severely hampered due to less understanding on host-pathogen interaction and also in-ability to detect the sub-clinical cases accurately (Pahangchopi *et al.,* 2014). So, farmers, advisers and supply chain actors should be involved in design and delivery of control programs to account for divergent approaches and levels of information (Morrison and Rose, 2023). At present, 'test and cull policy' and 'vaccination approach' are the two important tactics for JD control; and are used world-wide with variable success. The vaccination approach, however

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has limitation as it interfere with immunological tests (skin based hypersensitive test and ELISA) used for diagnosis of JD (Gupta *et al.,* 2019). Many vaccine based approaches also failing due to strain diversity of MAP, Rasper-Hossinger *et al*. (2023) reported that new MAP isolated typing data from 11 herds was showed intra-herd diversity of genotypes indicate a heterogeneity of MAP. Recently, we developed an'indigenous vaccine' that showed high protective efficacy against JD (Singh *et al.,* 2015). However, due to un-availability of test that can differentiate between infected and vaccinated animals (DIVA), vaccination at large scale could not be implemented so far.

ELISA based detection of anti-MAP antibodies is most preferred and sensitive test used for herd screening. However, the ELISA developed by whole cell protoplasmic antigen is reported to have lower specificity (Singh *et al.,* 2007a). They reported the specificity of whole cell protoplasmic 'indigenous ELISA' (i_ELISA) as 86%, though it was superior over commercial assay. Cho *et al.,* (2006) reported that as compared to cellular proteins, cultural filtrate proteins (CFPs)/ secretary proteins of MAP exhibited superior reactivity with positive serum samples of MAP. Further, the use of CFPs improved the sensitivity of ELISA over other commercial ELISAs for low MAP shedding in cattle (Shin *et al.,* 2008). However, the selection of specific protein/antigen (among different CFPs) remain a key challenge in diagnosis of MAP infection, since not a single antigen of MAP that is recognized by all stages of infected animals in different stages (early, sub-clinical and clinical) of disease. We hypothesized that use of multiple CFPs will not only enable us to detect the disease of different stages but will also facilitate in differentiating infected animals from vaccinated and healthy ones. To test this hypothesis, we developed a 'cocktail ELISA' (Recombinant proteins based ELISA 'rELISA') using 6 MAP specific CFPs (MAP 1693c, MAP 2168c, MAP Mod D, MAP 85c, MAP Pep AN and MAP Pep AC); and the DIVA potential of rELISA was further assessed and compared with i_ELISA.

MATERIALS AND METHODS

Study animals, classification and sampling

For the field studies of rELISA vaccination was done in four Goat herds of two states [Uttar Pradesh (UP) and Madhya Pradesh (MP)]. A total of 557 goats were included in this study. Goats were of variable age mainly from females and they have mixed physical conditions and 30-35% of the goats were suffering from clinical to advance clinical JD. All these goats were ear-tagged for identification. Of the total 557goats in four herds, 194 goats were sort out where, 127 goats having good health and physical conditions and they are negative by fecal microscopy, fecal PCR and Indigenous ELISA and 67 goats were weak, emaciated and had clinical sign of JD, Animals looked diseased with poor physical conditions and were positive by fecal microscopy, fecal PCR. Of these 127 healthy goats, 65 were vaccinated and under observation, 62 were in Healthy category and taken as negative control (Healthy / non-infected cattle), respectively. Samples of fecal and serum were driven from all four goat herds (Table 1).

Following six months post vaccination, all goats (of 3 groups) were re-sampled and re-tested using microscopy, i_ELISA and also by newly developed rELISA. The tests were performed as per method described below.

Smear Microscopy

Smear preparation from faces and further Ziehl Neelsen staining was performed as per method described earlier as per Singh *et al.,* (2010). After observing 100 fields of smear under microscope sample was graded into +1 (10 bacilli or one bunch), +2 (10 bacilli or one bunch in alternate of 2, 3 or 4 field), +3 (10 bacilli or one bunch in each alternate field), +4 (10 bacilli or one bunch in each field).

Indigenous ELISA (i_ELISA)

The i_ELISAwas developed earlier by using semi-purified whole cell protoplasmic antigens from the novel biotype ('Indian Bison Type') of MAP strain 'S 5' (Singh *et al.,* 2007b). The test was performed by following the method described by Singh *et al.,* (2007b). Optical densities (OD) were expressed as sample-to-positive (S/P) ratios as per Collins (2002) by following calculations.

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

Serum samples from culture positiveand negative goats were used as positive and negative controls, respectively. Based on S/P ratios, animals were categorized as 'Negative' (0.00-0.09), 'Suspected' or 'Borderline' (0.10-0.24), 'Low positive' (0.25-0.39), 'Positive' (0.40-0.99), 'Strong positive' (1.00-10.0). To achieve qualitative result into 'positive' and 'negative' term and also as per recommendation in earlier study (Chaubey *et al.,* 2019a), animals with S/P ratio as 0.00-0.39 (Negative + Suspected + Low Positive) were considered as 'Negative'; whereas, animals with S/P ratio ≥ 0.40 (Positive + Strong positive) were considered as 'Positive'.

Recombinant Proteins based ELISA (rELISA)

Earlier, 14 CFPs of MAP have been demonstrated to have impending investigative assessment for JD (Cho *et al.,* 2007). Of these, 6 CFPs proteins (MAP 1693c; MAP 2168c; MAP ModD; MAP 85C; MAP Pep AN and MAP Pep AC) those reacted strongly with polyclonal rabbit antibodies were selected in this study. All six MAP specific CFPs were cloned, expressed and purified by method described by Chaubey *et al.* (2018; 2019b). Using six recombinant CFPs rELISA was developed and used for screening as per (Chaubey *et al.,* 2019b).

'Indirect ELISA' was developed to detect IgG antibodies against cocktail of the recombinant antigen(s) (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, Germany) were coated with 100 µL of cocktail of recombinant secretary proteins containing 1µg of each of 6 antigens diluted in 10 mL antigen coating buffer. Coated plates were incubated overnight at 4°C. After incubation, antigen-coated plates were washed one time with washing buffer (1× 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 gm BSA in 20 mL of 1 × 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-labelled anti-species whole IgG antibody produced in rabbit at the dilution of 1:4000 in 1 × PBS. 100 µL of secondary anti-species antibody was added to each well and incubated for 50 minutes at 37°C. After four times washing, 100 µL of chromogenic substrate solution of O-Phenylenediamine dihydrochloride (OPD) (Cat. No. P3804, Sigma-Aldrich, Inc) was added to each well. Plates were incubated for 10-15 minutes in the dark at 37°C. Extent of the colour development (optical density) was measured at the absorbance of 450 nm using Bio-RADimark ELISA plate reader.Serum samples from infected and healthy animals were used for the optimization of assay.

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

$$\text{S/P ratio} = \frac{(\text{Sample OD} - \text{NegativeOD})}{(\text{Positive OD} - \text{NegativeOD})}$$

S/P ratios and corresponding status of recombinant cocktail secretary proteins-based ELISA in animals was determined.

Data analysis

All the three tests applied in this study were compared for their positivity rate (in percentage).

RESULTS AND DISCUSSION

On re-testing of total 194 goats ('healthy and non-vaccinated'- 62; 'healthy and vaccinated'- 65; 'infected'- 67), 75(38.7%), 124 (63.9%) and 67 (34.5%) goats were detected as positive by smear microscopy, i_ELISA and rELISA, respectively. Eight goats, though detected earlier as positive by both microscopy and i_ELISA, on repeat testing they were found negative in both i_ELISA and rELISAbut remain positive in smear microscopy. Highest agreement was found between 'microscopy and rELISA' (PA=95.9%; kappa score=0.911±0.031) followed by 'microscopy and i_ELISA' (PA=78.7%; kappa score=0.591±0.056) and 'i_ELISA and rELISA' (PA=66.5%; kappa score= 0.370±0.057). Comparative and stratified test results by the three tests are described in Table 2.

Table 1: Field trial of DIVA based rELISA in goats.

Livestock farms and place	Infected(*n*)	Healthy (*n*)	
		Vaccinated	Not vaccinated
Goat farm, CIRG, Mathura, UP	22	20	18
Goat farm, Garhi, Churmura, Mathura, UP	12	10	9
Goat farm, Sundrel, Dhar, MP	14	14	17
Kurkunda Farm, Kurkunda, Mathura, UP	19	21	18
Sub-total A	67	65	62

n- number; UP- Uttar Pradesh, MP- Madhya Pradesh, CIRG- Central institute for research on goats.

Table 2: Comparative test results of smear microscopy, i_ELISA and rELISA.

Microscopy		i_ELISA, *n*					rELISA, *n*				
		Positive*		Negative#			Positive*		Negative#		
		SP	P	LP	S	N	SP	P	LP	S	N
4+	21	4	17	-	-	-	11	10	-	-	-
3+	26	-	26	-	-	-	-	26	-	-	-
2+	16	-	14	2	-	-	-	14	2	-	-
1+	12	-	6	6	-	-	-	6	6	-	-
Positives	75	4	63	8	-	-	11	56	8	-	-
Negatives	119	5	52	11	28	23	-	-	35	39	45
Total	194	9	115	19	28	23	11	56	43	39	45
		194	194								

n- number; *Goats under strong positive (SP) and positive (P) categories of S/P ratio were considered as 'Positive'; #Goats under low positive (LP), suspected (S) and negative (N) categories of S/P ratio were considered **as** 'Negative'

The comparative study of S/P ratio as determined by both the ELISAs showed that rELISA has better profiling capacity than i_ELISA. The number of goats under S/P ratio category of 'SP', 'P', 'LP', 'S' and 'N' by i_ELISA were further profiled into 3, 5, 3, 3 and 2 distinct S/P ratio categories, respectively by rELISA (Table 3). Results showed that majority of vaccinated goats (92.3%) fall under 'P' category of S/P ratio by i_ELISA, whereas these goats were profiled as 'LP' (53.8%), 'S'(40%) and 'N'(6.2%), respectively by rELISA (Table 3). Interestingly, all the 65 vaccinated goats were positive in i_ELISA but were negative in rELISA. In addition to this, the infected goats those were detected as positive by i_ELISA were also positive in rELISA. Taken together these observations, it is possible to differentially detect the vaccinated goats based on positive result in i_ELISA and negative result in rELISA (Table 4).

In absence of control programme in India, JD has emerged endemic in the domestic livestock population in the country. Vaccine is one of the most promising control strategies and many countries have reduced the significant disease incidence (Singh *et al.,* 2014c; 2015, Bastida and Juste, 2011; Stringer *et al.,* 2014). In 2005, first time India has developed the 'indigenous vaccine' to control the JD in the goat farms (Jamunapari, Barbari, Jakhrana and Sirohi) located at CIRG, Makhdoom. The vaccine was proven to be highly effective in different field trials and has shown preventive (Singh *et al.,* 2015) as well therapeutic effect. However, JD vaccine is confounding factor in diagnosis of MAP infection when whole cell protoplasmic antigen based ELISA test used (Singh *et al.,* 2015). Therefore, JD vaccine cannot be used in the field at large scale, unless we have marker test to differentiate between infected and vaccinated animals.

In this study, we employed two types of tests (rELISA and i_ELISA) using two different types of antigens. It was hypothesized that since 'indigenous vaccine' contains 'inactivated MAP bacilli' with only traces of CFPs, therefore rELISA developed using early secreting CFPs will not react or results into positive among 'vaccinated animals'. Moreover, secretory CFPs govern major impact on the development of DIVA based novel sero-diagnostic techniques with improved sensitivity and specificity (Shin *et al.,* 2004; Malamo *et al.,* 2006; Bannantine *et al.,* 2011, Deb and Goswami, 2011). Cho *et al.,* (2006) found that MAP infected sera of cattle reacted more strongly with the CFPs/ secretory antigens as compared to the antigens of intracellular origin.

In our study, the positivity rate of i_ELISA was highest (63.9%) in comparison to smear microscopy (38.7%) and rELISA (34.5%). In-agreement with our observation, superior sensitivity of whole cell protoplasmic antigen-based ELISA over smear microscopy has also been reported earlier (Chaubey *et al.,* 2015). Plausible explanation of this difference might be due to the fact that shedding of bacilli in feces generally occurs intermittently and during clinical stage. Whereas, antibodies against MAP infection develop more early and it can be detected at sub-clinical stage too (Chaubey *et al.,* 2016). i_ELISA detected all smear positive goats as positive except 8 goats that were positive in microscopy but negative in both the ELISAs. Although this discrepancy could not be investigated further, we reasoned false positivity in smear microscopy or infection due to other acid-fast bacilli.

The rELISA, though correlated well with microscopy (PA= 95.9%), prepared by recombinant cocktail of CFPs was lower in positivity rate as compared to i_ELISA. here, high positivity in i_ELISA should be cautiously interpreted as plausibility of false positivity could not be excluded. However, lower positivity rate of rELISA (in comparison with i_ELISA) might be due to selection of only a few CFPs that do not necessarily express/ secrete through-out the period of disease progression from early followed by sub-clinical and clinical stage of disease. Since, i_ELISA has a mix of a range of antigens it is expected to detect the disease at its various stages. Despite lower sensitivity, rELISA has shown improved sero-profiling and has resulted into more stratified S/P ratio of samples as compared to i_ELISA (Table 3). Moreover, majority of vaccinated goats and infected goats fall under 'P' category of S/P ratio by i_ELISA, but these were segregated well by rELISA into different S/P ratio

Table 3: Comparative sero-status of goats (of different groups) tested by i_ELISA and rELISA tests.

Sero-status ini_ELISA, *n* (%)			Sero-status in rELISA						
			Positive* 67 (34.5)			Negative# 127 (65.5)		Groups	*n* (%)
			SP	P	LP	S	N		
Positive*	SP	4	2	2	-	-	-	Infected	
132		5	-	-	5	-	-	Healthy and	Infected: 67 (34.53)
(68.04)	P	60	-	-	30	26	4	vaccinated	Healthy and
			63	9	54	-	-	-	vaccinated: 65 (33.5)
Negative#	LP	11	-	-	6	3	2	Infected	Healthy and non
62	S	28	-	-	2	5	21	Healthy and	-vaccinated: 62 (31.9)
(31.9)	N-	23	-	-	-	5	18	non-vaccinated	

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

Table 4: S/P ratio range observed for different categories of animals in rELISA.

S/P ratios	Status of animal
≤0.09	Negative/Healthy
<0.40	Vaccinated/Suspected
≥0.40	Infected

categories. Improved sero-diagnosis with culture filtrate (CF)/ secretory proteins have also been reported earlier for MAP infection and also for other mycobacterial pathogens, including *M. bovis* and *M. tuberculosis* (Samanich *et al.* 2000; Waters *et al.,* 2006; Bannantine *et al.,* 2008; Wadhwa *et al.,* 2012). Karappusamy*et al.* (2018) have identified 15 proteins as product of the genes *sdhA*, *fadE25_2*, *mkI*, *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. De Souza *et al.* (2018) identified the Apa protein which is secreted by MAP as a novel faecal biomarker for diagnosis of MAP infection in cattle.

CONCLUSION

On careful analysis, we observed that most of the vaccinated goats showed differentially lower titer than infected goats in rELISA whereas there was no significant difference was in detecting 67 infected goats by both the ELISAs. However, sample testing by rELISAalone has limitation as it can't differentiate between vaccinated and non-infected goats. Therefore, for using the rELISA as DIVA test, serum must be tested first by i_ELISA followed by repeattesting of goats (especially those falling under 'P' and 'SP' categories), by rELISA.

This study indicates rELISAin combination with i_ELISAis able to differentiate infected, vaccinated and healthy animals; thus, may be used as companion marker assay for future JD vaccination program. rELISAhas also high specificity and sensitivity and has well correlated with clinical condition. However, further large-scale field validation of this assay is required in different herds, at different post-vaccination time points and also in different livestock species.

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Authorship contribution statement

Chaubey KK: designed, performed research and wrote paper; Singh PK: Analyzed and interpreted, Singh SV: proof reading; Deen Dayal: Referencing; S Y Mukartal: Revision; K Agrawal- Revision and proof reading.

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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Conflict of interest

The authors have no conflict of Interest.

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