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Diagnostic Potential of Three Antigens from Geographically Different Regions of the World for the Diagnosis of Ovine Johne's Disease in India

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ABSTRACT

Cross reactivity of three antigens of *Mycobacterium avium subspecies paratuberculosis* with sera of sheep endemic for Johne's disease was evaluated. Out of 40 sheep tested by fecal microscopy, 72.5% were shedding MAP. Using protoplasmic antigens (PPA) from three MAP strains isolated from different livestock species and geographical regions, 90, 77.5 and 2.5% sheep were positive in goat (Indigenous g-ELISA) and cattle (b-ELISA) based ELISA kits and ELISA kit for small ruminant (sr-ELISA), respectively. Only 2.5 and 10% sheep were positive and negative in all the four tests. Native species specific (goat origin novel 'Indian Bison Type' MAP) semi-purified whole cell PPA based ELISA (Indigenous g-ELISA) was superior in reacting with sera of native sheep than the commercial PPA of bovine origin (Allied Monitor Inc., USA) and commercial ELISA kit for small ruminants (ID Vet, France). Lower cross reactivity of antigens originated from US and France emphasized the need to develop tests based on local strain of MAP than strains from different livestock species and geographical regions. This is an important finding against the use of 'Global kits' without validating in local conditions. Study showed that kits developed from local strains of MAP were not only superior but also cost effective and will significantly contribute in programs for the control of JD in native sheep population.

Key words: *Mycobacterium avium* subsp., *paratuberculosis*, AFBs, Indigenous g-ELISA, b-ELISA, sr-ELISA, OJD

INTRODUCTION

Johne's Disease (JD) is chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp., *paratuberculosis* (MAP) infection. Clinical symptoms of disease in domestic livestock are non-specific (progressive weight loss, diarrhea, sub-fertility, infertility, emaciation, loss in productivity and death. Johne's disease is endemic in sheep population of the country and presence of MAP has been reported continually in long-established sheep farms (Singh *et al*., 2012).

Ovine JD has been recognized as major disease causing high economic losses to sheep and wool industry globally, USA (Bush *et al*., 2006; Pithua and Kollias, 2012; Menzies, 2010), Canada (Bauman *et al*., 2014), European countries (Martin and Aitken, 1991; Stau *et al*., 2012; Bush *et al*., 2005; Liapi *et al*., 2011), Japan (Nakamatsu *et al*., 1968), New Zealand (Norton *et al*., 2010), Australia (Bush *et al*., 2005) and India (Singh *et al*., 2014; Gupta *et al*., 2012; Sonawane and Tripathi, 2013; Vinodhkumar *et al*., 2013; Rawat *et al*., 2014).

Control of disease is hampered due to lack of indigenous, accurate and cost effective diagnostic tests. Low sensitivity and specificity of the diagnostic tests has been key obstacle in the success of traditional 'Test and cull method' (Kudahl *et al*., 2007) for the control of disease in domestic livestock species (Lu *et al*., 2008). Hence, good quality indigenous diagnostic tests are the key for the management and control of disease (Whittington and Sergeant, 2001) and prevent human infection through food chain. The ELISA is the most widely used test for the diagnosis of ovine JD (Singh *et al*., 2014) and can be easily adopted as mass or flock 'Screening test'. Based on the experience of corresponding author, it was hypothesized that antigenic variations in MAP strains originating from different livestock species and geographic regions may be major limiting factor in search of 'Universal ELISA kit'. Therefore, indigenous ELISA kit based on novel native strain (S 5) of MAP ('Indian Bison Type') was evaluated for cross reactivity (diagnostic potential) with two other antigens; commercial PPA of bovine origin (Allied Montior Inc., USA) and pre-coated PPA in ELISA kit for small ruminants (ID Vet, France) with sera of sheep endemic for Johne's disease.

MATERIALS AND METHODS

Samples: Faecal and serum samples were collected from 40 sheep (37 Muzaffarnagri, 3 non-descript) from sheep farms belonging to Central Institute for Research on Goats, Makhdoom and farmer's flock in Mathura district in the year 2014.

Shedding of MAP in feces (Microscopy): Faecal samples were screened by microscopy for shedding of MAP bacilli. Briefly, 2 g of faecal sample collected directly from each sheep were homogenized finely in tap water and concentrated by centrifugation at 4500 rpm for 45 min at room temperature. Smears prepared from the middle layer were heat fixed, stained with Ziehl-Nielsen (ZN) method and examined under microscope for pink colour (acid fast) short rods indistinguishable to MAP (Singh *et al*., 2013a).

Cross (Sero)-reactivity (ELISA kits): Serum samples were screened by three types of diagnostic ELISA assays using antigens from different livestock species and geographical regions of the world. The antigens were compared to estimate cross reactivity with serum of sheep flocks (farm and farmer's) endemic for ovine JD. One of the antigen used in 'Indigenous goat based ELISA' (g-ELISA) was derived from MAP ('Indian Bison Type') 'S 5' strain isolated from terminally sick goats located in Central Institute for Research on Goats (CIRG), the same campus where Muzaffarnagri sheep were located. Second antigen (commercially available from Allied Monitor Inc., USA) and used in 'Bovine based ELISA' (b-ELISA) was derived from MAP strain of bovine origin and third antigen was available as pre-coated plates in the ELISA kit provided by ID Vet, France for small ruminants (sr-ELISA), wherein exact source species was not mentioned by kit manufacturer (sr-ELISA).

Types of antigens

Semi-purified protoplasmic antigen from native MAP isolate (S 5) of goat origin: Semi-purified Protoplasmic antigen (sPPA) prepared from native 'Indian Bison Type' strain (S 5) of MAP isolated from a terminal case of JD in a goat (Sevilla *et al*., 2005) was used to develop an 'Indigenous ELISA' kit for screening of goats (Indigenous g-ELISA) (Singh *et al*., 2007a). The antigen (sPPA) was standardized at 0.1 µg per well of the microtiter plate. Serum samples and anti-goat horseradish peroxidase conjugate (Sigma Aldirch, USA) were used in 1:50 and 1:3000 dilution, respectively. Serum samples from culture positive and negative goats were used as positive and negative controls, respectively. Optical Densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per the method of Collins (2002) (Table 1).

Analysis of OD (absorbance) values:

$$\text{S/P ratio value} = [(\text{Sample OD} - \text{Negative OD})/(\text{Positive OD} - \text{Negative OD})]$$

Samples in positive (P) and strong positive (SP) categories were taken as positive for MAP infection. Sensitivity and specificity of 'Indigenous ELISA kit' as per (Singh *et al*., 2007b) was 83.3 and 90.0%, respectively.

Purified Protoplasmic Antigen (PPA) of MAP of bovine origin: Commercially available Purified Protoplasmic Antigen (PPA) of bovine origin was procured from Allied Monitor Inc., (USA) and used as per specifications of the manufacturer. Protein estimation of the dilution of antigen used, showed that 100 µL of coating buffer contained 0.4 µg per well of the microtiter plate (b-ELISA) and other steps were like 'Indigenous ELISA kit' (Singh *et al*., 2007b).

Purified Protoplasmic Antigen (PPA) of MAP (for small ruminants): The ELISA kit provided by ID Vet, France was for use in small ruminants (sr-ELISA) and was used as per the instructions of manufacturer. Pre-coated ELISA plate with PPA was provided by ID-VET. Positive and negative controls and test sera samples (sheep) were diluted in 96 well dummy plate with buffer 6 in ratio of 1:11 and samples were incubated after transferring 100 µL of diluted test samples to each well. After incubation plates were washed 3 times with 300 µL of 1X wash solution. Anti-ruminant IgG peroxidase conjugate (10X) was diluted to 1X in dilution buffer 3 and transferred 100 µL to each well, incubated and were again washed 3 times with 300 µL of the 1X wash solution. Substrate solution of 100 µL was added to each well and incubated for 15 min at 21°C and after adding stop solution, OD values were taken at 450 nm. Sensitivity and specificity of ID vet ELISA kit was reported as 42.0 and 99.0% (Kohler *et al*., 2008).

Interpretation: Mean OD values of samples were converted to S/P percentage using following formula (Table 2):

Table 1: Sample to positive ratios and status of Johne's disease on the basis of likelihood ratio

S/P Ratios	Johne's disease status
0.00-0.09	Negative
0.10-0.24	Suspected or borderline
0.25-0.39	Low positive
0.4-0.99	Positive
1.0-10.0	Strong positive

Table 2: S/P percentage and status of Johne's disease

S/P (%)	Status of Johne's disease
≤60%	Negative
≥60% to ≤70%	Doubtful
≥70%	Positive

Table 3: Statistical analysis between different test by Mc-Nemar test and Kappa agreement

	p-values				
Comparison of tests	Status	Values	Kappa	Strength of agreement	Confidence interval (95%)
Microscopy vs. indigenous g-ELISA	Significantly different	0.233	0.453	Moderate	0.145-0.761
Microscopy vs. b-ELISA	Not significantly different	0.7728	0.203	Fair	-0.127-0.533
Microscopy vs. sr-ELISA	Extremely significantly different	<0.0001	0.019	Poor	-0.020-0.058
Indigenous g-ELISA vs. b-ELISA	Not quite significantly different	0.0736	0.554	Moderate	0.226-0.881
Indigenous g-ELISA vs. sr-ELISA	Extremely significantly different	<0.0001	0.006	Poor	-0.015-0.045
b-ELISA vs. sr-ELISA	Extremely significantly different	<0.0001	0.015	Poor	-0.007-0.018

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

$$S/P\ (\%) = [(\text{Sample OD} - \text{Negative OD})/(\text{Positive OD} - \text{Negative OD})] \times 100$$

Statistical analysis: Mc Nemar's test and kappa agreement were applied to estimate level of significance between two tests using GraphPad software, USA (Table 3).

RESULTS

Out of the 40 sheep screened by microscopy and ELISA (Indigenous g-ELISA, b-ELISA and sr-ELISA), 1 (2.5%) and 4 (10.0%) sheep were positive and negative, respectively. Out of the 4 tests used, highest numbers of sheep (90.0%) were detected by indigenous g-ELISA followed by b-ELISA (77.5%), microscopy (72.5%) and sr-ELISA (2.5%). Sheep detected positive in microscopy (72.5%) and b-ELISA (77.5%) were also found positive in indigenous g-ELISA. In two tests combinations; microscopy and indigenous g-ELISA, microscopy and b-ELISA, microscopy and sr-ELISA, indigenous g-ELISA and b-ELISA, indigenous g-ELISA and sr-ELISA and b-ELISA and sr-ELISA, 29(75.0%), 24(60.0%), 1(2.5%), 31(77.5%), 1(2.5%) and 1 (2.5%) sheep were positive in both the tests, respectively. However, in the three ELISA kits, sr-ELISA had lowest sensitivity as compared to indigenous g-ELISA and b-ELISA (Table 4 and 5). Table 1 and 2 shows the cut off values (S/P ratios) recommended by each of the ELISA kit provider. Table 6 shows rate of shedding of MAP in feces in different categories [S/P ratios; Strong positive (SP), Positive (P), Low Positive (LP), Suspected (S), Negative (N)] with respect to status of MAP infection in 'indigenous g-ELISA test'. Microscopy had strong correlation with positive reactors (SP and P) in 'indigenous g-ELISA'. Microscopy exhibited the usefulness, since it detected 10% of the low positive sero-reactors and suspected sheep but had lower sensitivity as compared to 'indigenous g-ELISA', since 22.5% sheep positive in indigenous g-ELISA were negative in microscopy (Table 6).

Sensitivity and specificity of microscopy with respect to three ELISA tests: Microscopy with respect to indigenous g-ELISA, b-ELISA and sr-ELISA had 80.5, 77.4 and 100% sensitivity and 100, 44.4 and 28.2% specificity, respectively (Table 7).

Statistical analysis (Mc-Nemar test and kappa agreement): The p-value and kappa agreement for six different combinations (microscopy vs. indigenous g-ELISA, microscopy vs. b-ELISA, microscopy vs. sr-ELISA, indigenous g-ELISA vs. b-ELISA, indigenous g-ELISA vs. sr-ELISA and b-ELISA vs. sr-ELISA) were 0.233, 0.7728, <0.0001, 0.0736, <0.0001 and <0.0001 and

Table 4: Comparision of microscopy, indigenous g-ELISA, b-ELISA and sr-ELISA for the diagnosis of MAP infection (n = 40)

	Combinations					
Tests	1	2	3	4	5	Cumulative total n (%)
Microscopy	+	-	+	-	+	29 (72.5)
Indigenous g-ELISA	+	-	+	+	+	36 (90.0)
b-ELISA	+	-	-	+	+	31 (77.5)
sr-ELISA	+	-	-	-	-	01 (2.5)
n (%)	1 (2.5)	4 (10.0)	5 (12.5)	7 (17.5)	23 (57.5)	

Table 5: Status of MAP infection with respect to anti-MAP antibodies by three antigens (PPA) in indigenous g-ELISA, b-ELISA and sr-ELISA assays (n = 40)

	Status n (%)					
Tests	SP	P	LP	S	N	F
Indigenous g-ELISA	10 (25.0)	26 (65.0)	2 (5.0)	2 (5.0)	0	0
b-ELISA	11(27.5)	20 (50.0)	6 (15.0)	3 (7.5)	0	0
sr-ELISA	1 (2.5) (Positive)		0 (Suspected/doubtful)		39 (97.0) (Negative)	

n: Number, SP: Strong positive, P: Positive, LP: Low positive, S: Suspected, N: Negative, F: False

Table 6: Status of shedding of MAP by microscopy with respect to indigenous g-ELISA (n = 40 samples)

	Indignousg-ELISA status n (%)						
	SP	P	LP	S	N	F	
Tests	10 (25.0)	26 (65.0)	2 (5.0)	2 (5.0)	0	0	Total n (%)
Microscopy status n (%)							
3+	0	1 (2.5)	0	0	0	0	1 (2.5)
2+	2 (5.0)	4 (10.0)	0	0	0	0	6 (15.0)
1+	4 (10.0)	18 (45.0)	0	0	0	0	22 (55.0)
Total positives	6 (15.0)	23 (57.5)	0	0	0	0	29 (72.5)
Negative	4 (10.0)	3 (7.5)	2 (5.0)	2 (5.0)	0	0	11 (27.5)

n: Number, SP: Strong positive, P: Positive, LP: Low positive, S: Suspected, N: Negative, F: False

Table 7: Sensitivity and specificity of four diagnostic tests for the detection of MAP infection in sheep

Test combinations	TP	FP	TN	FN	Sen and Sp (%)
Microscopy vs. indigenous g-ELISA	29	0	4	7	Sen-80.5, Sp-100
Indigenous g-ELISA vs. microscopy	29	7	4	0	Sen- 100, Sp-36.3
Microscopy vs. b-ELISA	24	5	4	7	Sen-77.4, Sp- 44.4
b-ELISA vs. microscopy	24	7	4	5	Sen-82.7, Sp-36.6
Microscopy vs. sr-ELISA	1	28	11	0	Sen-100, Sp-28.2
sr-ELISA vs. microscopy	1	0	11	28	Sen-3.4, Sp-100
Indigenous g-ELISA vs. b-ELISA	31	5	4	0	Sen-100, Sp-44.4
b-ELISA vs. indigenous g-ELISA	31	0	4	5	Sen-86.1, Sp-100
Indigenous g- ELISA vs. sr-ELISA	1	35	4	0	Sen-100, Sp-11.4
sr-ELISA vs. indigenous g-ELISA	1	0	4	35	Sen-2.7, Sp-100
b-ELISA vs. sr-ELISA	1	30	9	0	Sen-100, Sp-23.0
sr-ELISA vs. b-ELISA	1	0	9	30	Sen-3.2, Sp-100

TP: True positive, FP: False positive, TN: True negative, FN: False negative, Sen: Sensitivity, Sp: Specificity

0.453, 0.203, 0.019, 0.554, 0.006 and 0.015, respectively. Strength of agreement was moderate, fair, poor, moderate, poor and poor, respectively and 95% confidence interval were 0.145 to 0.761, -0.127 to 0.533, -0.020 to 0.058, 0.226 to 0.881, -0.015 to 0.045 and -0.007 to 0.018, respectively in all 6 combinations tests (Table 3). Agreement between microscopy and b-ELISA was fair and was moderate in microscopy and indigenous g-ELISA and indigenous g-ELISA and b-ELISA.

DISCUSSION

For diagnosis of MAP infection two types of tests, organism based and antibody based were used. Using 4 assays showed that bio-load of MAP was very high (90%) in sheep screened, which

may be due to screening of suspected animals. Ovine JD has been reported to be endemic in sheep flocks in India. Singh *et al*. (2014) reported 32.7% bio-load in sheep population of the country in the survey of last 28 years (1985-2013). Microscopy exhibited its value by detecting 72.5% of sheep positive and none was positive independently in microscopy. Singh *et al*. (2013b) earlier also reported similar high bio-load (33.4%) in sheep using microscopy. Microscopy was simple, easy to perform, repeatable and cost effective and can be adopted as 'Flock screening test'. Using 3 antigens from geographically distinct regions of the world showed that it was prudent to use species specific antigen prepared from native strain (S 5) of MAP, the novel dominant bio-type ('Indian Bison Type') reported first time in the world from India (Sohal *et al*., 2009; Singh *et al*., 2013a). Similar findings have been reported in our earlier studies (Singh *et al*., 2007b). Present study also used semi-purified protoplasmic antigen from strain 'S 5' of MAP of goat origin in 'Indigenous g-ELISA' which detected highest percentage (90.0%) of sheep as positive and sheep was positive independently in the test. Our past 27 years studies (1985-2013) showed sheep and goat population of the country has been infected exclusively with this dominant bio-type ('Indian Bison Type') and has not been reported so far outside India. Sheep detected positive in 'Indigenous g-ELISA' were also detected by other tests in 2 and 3 tests combinations. Previous studies by Singh *et al*. (2007b) using Purified Protoplasmic Antigen (PPA) from Allied Monitor Inc., USA exhibited poor cross reactivity with sheep sera. However, in the present study purified PPA of bovine origin from Allied Monitor Inc., USA (supplied by ID Vet, France) exhibited better sensitivity and detected 77.5% sheep positive. These positive sheep were also detected by microscopy, indigenous g-ELISA and sr-ELISA. This may be due to change in the strain of the MAP used in making purified PPA by Allied Monitor Inc., USA, for this study PPA of Allied Monitor Inc., USA was supplied by ID Vet, France along with their ELISA kit on our request. The PPA used in ELISA kit (sr-ELISA) supplied by ID Vet was for goats and sheep and it had poor cross reactivity with anti-MAP antibodies in native sheep population. The strain used by ID Vet for making ELISA kit may be distinct from 'Indian Bison Type'.

Serum ELISA is fast and rapid 'Mass screening test' in livestock population. Studies time to time examined diagnostic efficacy of different antigens. Ferreira developed PPA-ELISA and compared sensitivity in relation to Herd Check (commercial ELISA) as 47.5% and specificity 86.8 (Ferreira *et al*., 2002). Speer *et al*. (2006) evaluated SELISA with commercial ELISA where sensitivity and specificity of SELISA was superior to commercial ELISAs for routine diagnosis of JD (Speer *et al*., 2006). Like our study in sheep, JTC-ELISA developed by Shin *et al*. (2008) was particularly sensitive at detecting low level fecal shedders of MAP (sub-clinical infection) and works effectively both on serum and milk samples for the detection of cattle infected with sub-clinical MAP infections, providing a cost-effective diagnostic tool to support JD control programs in cattle herds. Unlike Cho and Collins (2006) reporting that protein antigens of sero-diagnostic potential were more abundant in culture filtrates than cellular extracts from liquid culture of MAP, they used protein based species specific semi-purified whole cell PPA from cellular part of novel native MAP biotype (Indian Bison Type) cultured on solid HEY medium and was highly sensitive and specific (Singh *et al*., 2007b, 2009a). Kumar *et al*. (2006) reported that use of species specific antigens from 'Indian Bison Type' MAP showed better cross reactivity i.e., using PPA from sheep isolate in sheep rather than goat origin PPA in sheep. However it may not be practical approach, therefore PPA from 'Indian Bison Type' has been evaluated to estimate cross reactivity in sheep, goats, cattle and buffaloes (Singh *et al*., 2014), where it has shown better cross reactivity as compared to PPA of foreign origin.

'Indigenous g-ELISA kit' based on antigen (PPA) of goat origin was developed in India using novel 'Indian Bison type' MAP' strain ('S 5') (Singh *et al*., 2007a). Kit had high sensitivity and specificity as compared to commercial ELISA kits (Singh *et al*., 2009b). Indigenous ELISA was optimally correlated with culture and was good for estimating the sero-prevalence (Singh *et al*., 2007c). Indigenous g-ELISA was superior than AGPT for the diagnosis of MAP infection in cattle (Pahangchopi *et al*. 2014). In another study, sensitivity and specificity of indigenous ELISA kit was 66.6 and 75.0% and 68.1 and 66.6% with tissue culture and tissue PCR, respectively (Singh *et al*., 2008). For estimating sensitivity and specificity of ELISA in case of MAP infection is by comparing serum ELISA with fecal culture (Garg *et al*., 2015). Similar comparative studies were carried out by Singh *et al*. (2009b), where 'Indigenous g-ELISA kit' had superior diagnostic potential and high specificity as compared to commercial ELISA kit.

CONCLUSION

Study showed that native species specific (goat origin 'Indian Bison Type' MAP) antigen based ELISA (Indigenous g-ELISA) was superior in reacting with sera of native sheep than the commercial purified PPA of bovine origin (Allied Monitor Inc., USA) and commercial ELISA kit for small ruminants (ID Vet, France). Lower cross reactivity of purified antigens of MAP originated from France (small ruminant kit) and USA (bovin) emphasized need to develop antigen antibody based tests from local strains of MAP. Results also indicated that level of MAP infection in native sheep population may be very high. Both 'Indigenous g-ELISA' and microscopy may be used for the diagnosis and control of ovine JD. 'Indigenous g-ELISA' being low-cost, easy to perform and sensitive can be used as 'Flock screening' test in Indian sheep population. This is an important finding and will significantly contribute in effective implementing JD control program in India.

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