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Research Article

Development of New 'Indigenous Dot-ELISA Kit' as Sensitive Field Based Herd Screening Test for the Diagnosis of Johne's Disease in the Domestic Buffalo Population

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Abstract

Johne's disease is endemic in the domestic riverine buffalo population of the country and bio-load of Mycobacterium avium subspecies paratuberculosis is increasing in the absence of indigenous diagnostic kits and control programs. A new 'dot-ELISA kit' has been developed and validated with indigenous plate ELISA for the screening of buffaloes against Johne's disease. Out of 156 serum samples screened 41.0 (64), 85.8 (134) and 85.2% (133) were positive for MAP infection by indigenous plate ELISA kit condition (A), condition (B) and indigenous dot ELISA, respectively. Dot-ELISA kit detected 85.2 (133) and 90.3% (141) buffaloes as positive together with indigenous plate ELISA kit in condition A and B, respectively. Comparison of 'Indigenous plate-ELISA' with 'Indigenous dot-ELISA' revealed substantial agreement between two tests. Study showed that 'Indigenous dot-ELISA test' has potential to be sensitive and cost effective 'Field based herd screening test' for the large scale screening of the domestic livestock population against Johne's disease. The study also showed that despite high slaughter rate, incidence of Johne's disease was high in native population of riverine buffaloes (Bubalus bubalis) and call for immediate control of disease.

Key words: *Mycobacterium avium* subsp., *paratuberculosis*, plate-ELISA, dot-ELISA, Johne's disease, herd screening test

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INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP), the cause of chronic incurable enteritis called as Johne's Disease (JD) primarily infects domestic livestock species and has also been recovered from wild ruminants (e.g., bison, blue bulls and deer) as well as human beings (Chiodini *et al*., 1984; Beard *et al*., 2001; Hermon-Taylor, 2009; Singh *et al*., 2010). Disease has major impact on the farm economy and has been frequently reported from dairy farms and farmer's herds worldwide (Kumar *et al*., 2007; Singh *et al*., 2013a, b; Singh *et al*., 2014a). Buffalo considered as 'Black Gold' is primarily found in Asia. Buffalo population in the country has risen from 43.40 million in 1951-109 million in 2013; according to FAO and majority (98.0%) of buffaloes in the region are raised by small farmers owning less than two hectares of land and less than five buffaloes. Sharp rise in human population has led to the high demand of cheap source of protein. In recent years there has been unprecedented rise in slaughter of low or unproductive buffaloes to meet the ever growing demand of food for internal consumption and export to middle East countries. Despite high slaughter rate, per animal productivity in case of buffaloes is low. This is mainly due to the presence of chronic infections, like Johne's disease. In the absence of 'Indigenous diagnostic kits', there are very few studies estimated status of Johne's disease in the riverine buffalo (*Bubalus bubalis*) population. In the absence of control programs at national scale, bio-load of MAP has increased continuously in the bovine (buffaloes and cattle) population (Singh *et al*., 2010, 2013a, 2014c). Transmission of MAP bacilli from infected animals to susceptible young buffalo calves cannot be prevented. Since MAP is passed from generations to generations through semen, during pregnancy, by feeding of colostrums and milk (Buergelt *et al*., 2006). Therefore, improved hygiene, good management practices, segregation of diseased animals and culling of infected animals has little impact on the overall management and control of disease in herds and flocks.

Lack of indigenous tests for the detection infected animals in early stages of the disease is the major stumbling block in the control of JD at National level (Singh *et al*., 2014b). In chronic infections like JD, it has been recommended to use multiple diagnostic tests (Singh *et al*., 2014a; Wadhwa *et al*., 2012). Serology has been used in sero-screening and diagnosis of MAP infection as stated by OIE in 2014. Johnin the only test for the diagnosis of disease in the field but suffers from poor sensitivity and specificity. Of the range of diagnostic tests (faecal microscopy, fecal culture, blood PCR, ELISA and IFN-γ) available, none has potential to be used in the field. 'Indigenous Plate-ELISA kit' initially standardized for the diagnosis of Johne's disease in goats (Singh *et al*., 2007a) was later adopted for the screening of cattle, buffaloes and sheep population (Singh *et al*., 2007b). The study standardized an indigenous 'dot-ELISA kit' (d-ELISA) for the screening of buffaloes for Johne's disease. Indigenous 'plate-ELISA kit' (p-ELISA) was used as 'Parallel test' to evaluate efficacy of d-ELISA. Study also evaluated two test combinations (p-ELISA and d-ELISA) for the diagnosis of JD in buffaloes as 'Herd screening test'.

MATERIALS AND METHODS

Serum samples were collected from native buffalo population of government farm located at Kiratpur (Itarsi) in Central India, Buffaloes were managed under semi-intensive system of management. Most of the buffaloes were weak and suspected for Johne's disease at the time of sampling. Some buffaloes had diarrhoea and clinical symptoms of Johne's disease. Native buffalo population sampled belonged to Murrah, Jafrawadi and Bhadawari breeds. A total of 156 buffaloes (153 females and 3 males) were sampled between March and September, 2015. Serum samples were collected randomly and screened for MAP infection using goat based 'Indigenous plate-ELISA' kit; standardized in buffaloes (Yadav *et al*., 2008) and the newly standardized indigenous 'dot-ELISA' kit using same semi-Purified Protoplasmic Antigen (sPPA). Buffaloes were maintained on optimal nutrition in semi-intensive management system (Green fodder, dry bhusa with mineral concentrate). Housing and hygienic conditions of the farm were good.

Indigenous plate ELISA (p-ELISA): Native strain (S5) of MAP characterized as 'Indian Bison type' of goat origin was used as antigen source (Sohal *et al*., 2009). Initially 'Indigenous p-ELISA' developed for goats (Singh *et al*., 2007a) has previously been standardized in cattle (Sharma *et al*., 2008) and buffaloes (Yadav *et al*., 2008). Soluble whole cell, semi Purified Protoplasmic Antigen (sPPA) was prepared from the strain (S5) of novel 'Indian Bison type' biotype of MAP (Strain S5), isolated from a terminal case of JD in a goat (Sevilla *et al*., 2005). Antigen was standardized at 0.1 μg in 100 μL of carbonate-bicarbonate buffer, (pH 9.6) was added in each well of flat bottom 96 well ELISA plate and incubated for overnight at 4°C. Plate was washed thrice with PBST (PBS with 0.05% tween

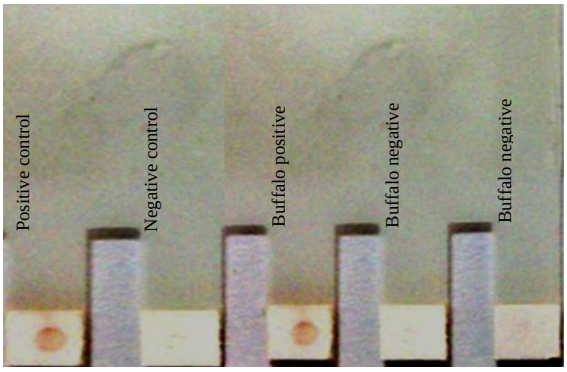

Fig. 1: Dot-ELISA test in buffaloes serum samples showing brown dot for positive samples for *Mycobacterium avium* subspecies *paratuberculosis*

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

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
0.40-0.99	Positive
1.00-10.0	Strong positive

20), blocked with 100 µL of 3% skimmed milk in PBS and was incubated at 37°C for 1 h. After blocking, plate was washed thrice with PBST, test serum samples (100 µL of 1:50 diluted samples) were added in duplicate wells and incubated for 2 h at 37°C. Washing of plate was done thrice, 100 µL of optimally diluted (1:2500) rabbit anti-bovine conjugate was added and plates were again incubated for one hour at 37°C. Washing was done five times with PBST, 100 µL of freshly prepared OPD substrate was added and incubated till development of colour (5-10 min) at 37°C. Absorbance was read at 450 nm in ELISA reader (i Mark micro-plate reader, Biorad). Serum samples from weak culture positive and healthy and culture negative buffaloes 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:

$$\text{S/P ratio value} = \frac{\text{Sample OD-Negative OD}}{\text{Positive OD-Negative OD}}$$

Values of sample to positive ratios and corresponding status of Johne's disease in animals was determined: In condition A, samples in Positive (P) and Strong Positive (SP) categories of S/P ratio were taken as positive for MAP infection. Whereas, in condition B, samples in Low Positive (LP), Positive (P) and Strong Positive (SP) categories of S/P ratio were taken as positive for MAP infection. Sensitivity and specificity of the 'Indigenous p-ELISA kit' as per Singh *et al*. (2007b) was 83.3 and 90.0%, respectively.

Indigenous dot ELISA (d-ELISA): Plastic strips with 12 legs (combs) with nitrocellulose membranes were coated with 1 µL (4 µg μL^{-1}) of antigen solution from strain 'S5' and incubated at 37°C for 2 h. Combs were stored at 4°C for further use. Subsequently, 250 µL of blocking solution (3.0% skimmed milk powder in PBS) was added to the used and cleaned ELISA plate wells and the strips were incubated at 37°C for 1 h. Combs were washed in PBST solution (3 times) and dipped in 250 µL of serum solution (1:50) incubated at 37°C for 1 h followed by washing in PBST (3 times). The 250 µL of rabbit anti-bovine HRP conjugate solution was added to each well and combs were incubated at 37°C for 30 min. Finally, 250 µL of 3,3´-Diaminobenzidine (6 mg/10 mL of 1X PBS) (Substrate solution) was added in new wells of used and cleaned ELISA plates, wherein combs were dipped till colour was developed (5-10 min) at room temperature. Reaction was stopped by dipping the combs in autoclaved triple distilled water air dried and observed for the appearance of brown dots (Fig. 1). The results of positive and negative controls at each comb were used to assist in reading of the test samples.

Statistical analysis: Mc Nemar's test and kappa agreement have applied for the measure the statistical significance between results of two tests (Table 2 and 3) by Graph Pad software, USA and sensitivity and specificity of the tests was measured by Med-Calc software, Belgium (Table 4).

RESULTS

Condition A: Out of 156 buffaloes screened, 41.0 (64) and 58.9% (92) were positive and negative in p-ELISA, respectively (Table 2). However, in d-ELISA, 85.2 (133) and 14.7% (23) buffaloes were positive and negative, respectively. On the basis of S/P ratio, 0.6, 40.3, 44.8, 11.5 and 2.5% buffaloes were in the strong positive, positive, low positive, suspected and negative categories with respect to the status of Johne's disease infection in p-ELISA. Results showed that with respect to the status of Johne's disease, 41.0 (64) and 14.7% (23) samples that were positive and negative in both the tests were

considered as True Positives (TP) and True Negatives (TN), respectively (Table 2). Proportional agreement between 'p-ELISA' and 'd-ELISA' was 55.7% (Table 2). However, there were 69 (44.2%) buffaloes as false positive p-value and kappa agreement were calculated to compare combinations of tests; p-ELISA vs d-ELISA and p-value was >0.0001 which was significantly different. Whereas, Kappa agreement was 0.215 and strength of agreement was fair and 95% confidence interval was 0.129-0.300 (Table 4).

Condition B: Out of 156 buffaloes screened, 85.8% (134) buffaloes were positive in p-ELISA kit as compared to 85.2% (133) d-ELISA. However, these 85.2% positive buffaloes were distributed in strong positive (0.6%), positive (40.3%), low positive (39.7%) and suspected (4.4%) categories of S/P ratio of p-ELISA kit. Therefore, of 156 buffaloes screened by p-ELISA and d-ELISA 126, (80.7%) and 15 (9.6%) buffaloes were considered as true positive and true negative, respectively. With a mis-match of 9.6% (15) buffaloes, of which 8 (5.1%) can be considered false negative and 7 (4.4%) buffaloes can be considered as false positive in d-ELISA. Similarly in d-ELISA 5.4% (8) buffaloes positive in p-ELISA were detected as negatives and can be considered as false positives in p-ELISA. However, 2.5% (4) negative samples in p-ELISA and d-ELISA matched perfectly. Proportional agreement between 'p-ELISA' and 'd-ELISA' was 90.3% (Table 3). However, two tests together detected, 133 (85.2%) buffaloes as positive (Table 3). The pvalue and kappa agreement were calculated for comparative combinations of tests (p-ELISA vs d-ELISA) and p-value was 1.0. Kappa agreement was 0.611 and strength of agreement was good and 95% confidence interval was 0.431-0.790 (Table 4).

Table 2: Comparative evaluation of indigenous plate-ELISA and dot ELISA in buffaloes (n = 156 samples)-Condition A

Tests		Indigenous p-ELISA status n (%)					
		Negative (N)	Suspected (S)	Low positive (LP)	Positive (P)	Strong positive (SP)	Total n (%)
		4 (2.5)	18 (11.5)	70 (44.8)	63 (40.3)	1 (0.6)	156
Condition A	Total	Negatives-92 (58.9)			Positives 64 (41.0)		
Dot ELISA status n (%)	Negative	4 (2.5)	11 (7.0)	8 (5.1)	0 (0.0)	0 (0.0)	23 (14.7)
	Total	N-23 (14.7) or TN			P- 0 (0.0) therefore FN		
	Positive	0 (0.0)	7 (4.4)	62 (39.7)	63 (40.3)	1 (0.6)	133 (85.2)
	Total	N-69 (44.2) or FP			P-64 (41.0) or TP		

*Figures in parenthesis are percent, Total samples (n) =156, N: Negative, P: Positive, TP: True positive, TN: True negative, FN: False negative, FP: False positive, Sensitivity: 100%, Specificity: 25%

Table 3: Comparative evaluation of indigenous plate-ELISA and dot ELISA in buffaloes (n = 156 samples)-Condition B

Tests		Indigenous p-ELISA status n (%)					
		Negative (N)	Suspected (S)	Low positive (LP)	Positive (P)	Strong positive (SP)	Total n (%)
		4 (2.5)	18 (11.5)	70 (44.8)	63 (40.3)	1 (0.6)	156
Condition B	Total	Negatives-22 (14.1)			Positives 134 (85.8)		
Dot ELISA status n (%)	Negative	4 (2.5)	11 (7.0)	8 (5.1)	0 (0.0)	0 (0.0)	23 (14.7)
		True negatives-15 (9.6)		False negative -8 (5.1)			
	Positive	0 (0.0)	7 (4.4)	62 (39.7)	63 (40.3)	1 (0.6)	133 (85.2)
	Total	False positive -7 (4.4)		True positives - 126 (80.7%)			

*Figures in parenthesis are percent, Total samples (n) =156, N: Negative, P: Positive, TP: True positive, TN: True negative, FN: False negative, FP: False positive, Sensitivity: 94.0%, Specificity: 68.0%

Table 4: Statistical analysis between two tests by Mc-Nemar test and Kappa agreement in buffaloes

Conditions	Comparison of tests	p-value				
		Status	Value	Kappa	Strength of agreement	95% confidence interval
Condition A (P+SP = Positive)	p-ELISA vs d-ELISA	Extremely significantly	>0.0001	0.215	Fair	0.129-0.300
Condition B (LP+P+SP = Positive)		Not significantly different	1.0000	0.611	Good	0.431-0.790

LP: Low positive, P: Positive, SP: Strong positive, Kappa value (0.0-0.20, poor, 0.21-0.40, Fair, 0.41-0.60, moderate, 0.61-0.80, substantial good and 0.81-100, perfect)

DISCUSSION

In India though first case of Johne's disease was reported in 1913 but it is still a major health problem in domestic ruminants (Singh *et al*., 2014a) and country-wide estimates on prevalence of MAP infection are not available. In a major study by Singh *et al*. (2014a), 28.3% bio-load of MAP infection was reported in buffaloes from North India in last 28 years (1985-2013). This study also revealed that at least 41.0% (64) buffaloes can be safely considered as positives for MAP infection using indigenous plate-ELISA kit, since these buffaloes were detected positive in two tests combinations (Table 2). However, a total of 133 (85.2%) and 23 (14.7%) buffaloes were positive and negative in dot-ELISA (Table 2). Since disease is endemic in domestic livestock (Singh *et al*., 2014a), low positive (44.8% in p-ELISA and 39.7% in d-ELISA) can be considered as positive. In a study from Agra region of North India, Yadav *et al*. (2008) detected MAP from 48.0% tissues of unproductive buffaloes slaughtered for meat production. Using 'Indigenous ELISA kit', they showed that sero-prevalence of MAP infection in slaughtered buffaloes was 46.7% in the Agra region. Another study by Singh *et al*. (2008), wherein large scale sero-survey was conducted using this p-ELISA kit, 28.6% in buffaloes from Northern India were found positive. Sero-prevalence was low (8.6-10.5%) in Murrah breed of young bulls from the states of Uttar Pradesh and Punjab using this p-ELISA kit. However, large number of bulls on borderline (78.0-84.2%) and were considered as negative. The comprehensive study of 28 years (1985-2013) by Singh *et al*. (2014a) showed that, bio-load of MAP was moderately high (28.6%) in buffaloes as compared to goats (20.1%) and was behind cattle (39.3%), sheep (32.7%). Lillini *et al*. (2002) reported 13.3% prevalence of MAP in the Latium region of Italy using PCR in fecal samples of water buffalo herds. Molecular epidemiology studies by Kaur *et al*. (2011) revealed that as compared to 'Cattle type' biotype, 'Indian Bison type' was the predominant (82.0%) biotype in domestic livestock including buffaloes. Georges *et al*. (2011) reported 13.1% of water buffaloes were serologically positive for MAP in ELISA and 13.2% were positive in IFN-γ test. They found significant association between age and sero-positive test results, (p = 0.007, chi square 1 df, 95% confidence). Sezzi *et al*. (2010) studied 1400 buffaloes belonging to 71 herds in the Latium region of Italy using two different commercial ELISA kits (Pourquier and ID.vet). In Pourquier kit none of the buffaloes was positive, whereas in ID.vet kit 3 buffaloes were positives (0.2% prevalence). Similarly, Singh *et al*. (2007a) showed that using commercial tests antigen the sensitivity of the ELISA was extremely low. It may be due to the use of different strains as the source of antigen. Desio *et al*. (2013) carried out a study on 1350 buffaloes belonging to 56 herds in the Caserta province, of Campania region, Italy. The prevalence of infected buffalo dairy herds was estimated by a commercial ELISA kit using individual blood samples of animals over 24 months of age. On the basis of performance (sensitivity 43%, specificity 99.3%) of ELISA test on serum, the resulting true prevalence at animal level and at herd level was 4% (95% CI 3-5%) and 74,1% (95% CI 71.8-76%). Waqas *et al*. (2015) reported 4,5% prevalence of MAP infection on the basis of suspected lesions in buffaloes with non-significant difference between age groups. However, prevalence was relatively higher in buffaloes more than 10 years old (6.1%) than buffaloes of less than 5 years (3.6%) of age and between 5-10 years (3.7%) years of age. Gamberale *et al*. (2014) reported bio-load of MAP in buffaloes over 12 months were subjected to yearly serological examination by ELISA and positive animals were culled. The overall yearly raw prevalence obtained was very low (1.0, 2.0-0 and 0%) between 2009-2012. Abbas *et al*. (2011) has screened the breeding and teaser bulls for the presence of antibodies against MAP at three Semen Production Units (SPUs) located in Punjab, Pakistan. A total of 253 samples were collected from SPUs and a commercially available indirect screen ELISA (Is-ELISA) was applied. Indirect screen-ELISA detected antibodies in 20 (24.6%), 16 (22.8%) and 17 (16.6%) samples from SPU-I, SPU-II and SPU-III, respectively. Collectively, seroprevalence of 20.0% (47/235) in breeding bulls and 33.3% (6/18) in teaser bulls was observed and thus it poses a potential threat of disease spread to a high number of heifers and cows through artificial insemination. Khan *et al*. (2010) has analysed cattle (*Bos taurus*) and buffalo (*Bubalus bubalis*) from an abattoir of the district of Lahore for the presence of MAP and *Mycobacterium bovis* through acid-fast

Table 5: Bio-load of Johne's disease in buffaloes and domestic livestock species globally and in India

Country	Species	Tests	Prevalence (%)	Reference
Global studies				
USA	Cattle	Dot-ELISA	65.9 (Non-absorbed serum)	Bech-Nielsen *et al.* (1993)
			34.1 (Absorbed serum)	
		Fecal Culture	91.4 (Non-absorbed serum)	
			97.8 (Absorbed serum)	
USA	Cattle	Culture	85.1	Woodruff *et al.* (1991)
		ELISA	79.7	
		AGID	50.4	
		Dot-ELISA	64.1	
Italy	Buffaloes	Fecal PCR	13.3	Lillini *et al.* (2002)
Italy	Buffaloes	ELISA	0.2	Sezzi *et al.* (2010)
Italy	Buffaloes	ELISA	4	Desio *et al.* (2013)
Italy	Buffaloes	ELISA	1.0, 2.0-0, 0	Gamberale *et al.* (2014)
Pakistan	Buffaloes	Suspected lesions	4.5	Waqas *et al.* (2015)
Pakistan	Buffaloes	ELISA	16.6-33.6	Abbas *et al.* (2011)
Pakistan	Buffaloes	Microscopy	16.4 (MLN)	Khan *et al.* (2010)
			17.4 (INT)	
		PCR	12.8 (MLN)	
			12.4 (INT)	
West indies	Buffaloes	IFN-γ	13.2	George's *et al.* (2011)
Indian studies				
India	Buffaloes	ELISA	21.3	Sivakumar *et al.* (2005)
India	Buffaloes	Culture	70.0	Sivakumar *et al.* (2006)
		PCR	30.0	
India	Buffaloes	Culture	48.0	
			34.0 (MLN)	Yadav *et al.* (2008)
			36.0 (INT)	
		ELISA	46.7	
India	Buffaloes	ELISA	28.6	Singh *et al.* (2008)
India	Buffaloes	ELISA	5.8	Mohan *et al.* (2009)
India	Buffaloes	ELISA	0.0	Tripathi *et al.* (2009)
India	Buffaloes	Microscopy	71.0	Kaur *et al.* (2011)
		PCR	55.0	
India	Buffaloes	Shedding	15	Chauhan *et al.* (2011)
India	Goats	Dot-EIA	18.4	Rajukumar *et al.* (2011)
		ELISA	23.8	
India	Buffaloes	ELISA	8.6-10.5	Singh *et al.* (2014b)
India	Buffaloes	Microscopy, culture, ELISA, PCR	28.3	Singh *et al.* (2014a)
India	Buffaloes	ELISA	56.0	Singh *et al.* (2014c)

staining and polymerase chain reaction. Most of the animals were emaciated. Diarrhea was noticed in 15.6% of buffaloes and 19.2% of cattle. Intestinal pathology was observed in 29% of buffaloes and 32.8% of cattle. Number of Mesenteric Lymph Nodes (MLN) showing gross lesions was a little higher (35.6%) in cattle than buffaloes (31.2%). Acid-fast staining of tissue scraping smears revealed the presence of Acid-Fast Bacilli (AFB) in 17.4% intestinal and 16.4% MLN tissue samples in buffalo while in cattle 19.2% intestinal and 17.8% MLN were found positive for AFB. In buffaloes, PCR confirmed 12.8% intestinal and 12.4% MLN positive samples for MAP. However, in cattle, PCR analysis demonstrated 14.2% positive results for MAP in both MLN and intestinal tissue samples. PCR also confirmed *M. bovis* in 5.8% of cattle and 5% of buffaloes MLN and intestinal tissues. PCR positive tissue samples for MAP were from those animals which were emaciated, having diarrhea and severe gross lesions. Acid Fast Bacilli were was also detected in tissue scrapping smears of these animals.

Mohan *et al.* (2009) reported 5.8% sero-prevalence of paratuberculosis in buffaloes from unorganized dairy farms in Gujarat. Incidence of MAP was lower in rural dairy population than organized dairy farms. Tripathi *et al.* (2007) screened 320 buffaloes (Central West India: 80, Northern India: 240) sera for MAP infection by Pourquier ELISA kit did not show antibody prevalence. Sivakumar *et al.* (2005) estimated sero-prevalence of MAP in buffalo to be 21.3% in Chennai. In another study, the Ziehl-Neelsen's stained tissue sections revealed acid-fast bacilli in grade-3 and grade-2 buffaloes and acid-fast granular debris were present in grade-1 buffaloes. Of 20 buffaloes, 14 (70%) were positive by IS900 PCR and 6 (30%)

by MAP culture (Sivakumar *et al*., 2006). Singh *et al*. (2014c) screened 25 young Murrah bulls, 14 (56.0%) were positive for BJD. Sero-incidence of BJD was higher in young bulls of Murrah breed in their native tract. Chauhan *et al*. (2011) given his assumption that annual death losses within MAP infected herd may reach 10% and incidence of subclinical cases shedding organisms intermittently may be as high as 15.0% (Table 5).

In an earlier study, Bech-Nielsen *et al*. (1993) evaluated dot-ELISA results using non-absorbed sera in 29 of 44 (65.9%) clinically-suspect animals giving positive results by faecal culture and 85 of 93 (91.4%) cattle testing negative by faecal culture. With absorbed sera, the sensitivity of visual determination decreased to 15 of 44 (34.1%) while specificity increased to 91 of 93 (97.8%). Approximately 75% of cattle yielding positive results by dot-ELISA were heavy bacterial shedders (>1,500 colonies/g of faeces) at the time of serological testing. Rajukumar *et al*. (2006) measured the sensitivity and specificity for the dot-EIA with respect to plate ELISA and found to be 65.6% and 92.9% and for plate ELISA with respect to dot-EIA were 78.0 and 89.0%, respectively. Woodruff *et al*. (1991) used serum dot ELISA for the screening of MAP in animals were found infected and non-infected by culture, where 86 were positive of 101 infected cattle and 64 were negative for 64 non-infected cattle. Results of conventional ELISA and Agar Gel Immune Diffusion (AGID) tests were positive for 79 of 99 and for 51 of 101 infected cattle, respectively. The dot ELISA also was evaluated by comparing results of testing 708 sera with results of bacteriologic culturing of matched fecal samples from 262 cattle in 3 central Ohio dairy herds known to include cattle infected with MAP. Results of the dot ELISA were positive for 25 of 39 sera from cattle with positive results on culturing of concurrently obtained fecal specimens. The dot ELISA results were negative for 661 of 669 sera from cattle with negative results to culturing of concurrently obtained fecal specimens. The 39 sera from cattle with positive results on bacteriologic culturing of matched fecal specimens had positive results for ELISA and the AGID test 25 and 14 times, respectively. The 669 sera from cattle with concurrently negative results on bacteriologic culturing of faeces had negative results to ELISA and the AGID test 559 and 668 times, respectively. However, the comparative studies involving d-ELISA and p-ELISA for the detection of MAP infection in buffaloes are not available in literature. Also the comparison between d-ELISA and p-ELISA between condition A and B showed strength of agreement in condition B was good and p-value did not differ significantly (Table 4).

CONCLUSION

Study showed that d-ELISA could be used as highly sensitive and cost effective test for the diagnosis of JD and for the large scale screening of buffalo herds. Sensitivity of d-ELISA was higher than p-ELISA, especially for low shedders (Condition B). Therefore, d-ELISA emerged as 'Field based herd screening test' which can be used for the implementation of JD control program in India with p-ELISA as parallel test or as stand alone test.

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