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Evaluation of 'cattle' and 'Indian Bison' type antigens of *Mycobacterium avium* subspecies *paratuberculosis* for diagnosis of Bovine Johne's Disease using 'indigenous ELISA' and AGPT

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Two antigens ('cattle' type and 'Indian Bison' type) of *Mycobacterium avium* subspecies *paratuberculosis* were evaluated for diagnosis of Johne's disease (JD) in a *gaushala* (cattle herd). Of the 160 cows of *Sahiwal* and *Hariana* breeds screened, 81 (50.6%) tested positive in ELISA and 66 (41.8%) in AGPT test. Using the two antigens, 33.5% tested positive in both the tests while 41.1% tested negative. Exclusively, only 8.2% tested positive in ELISA while 17.1% tested positive in AGPT. Two antigens together detected 58.9% prevalence of MAP in the *gaushala*. Individually, indigenous ELISA using antigen from native source of MAP proved superior to AGPT in the diagnosis of JD in cows.

Keywords: AGPT, BJD, ELISA, Indian cow, JD, Livestock, MAP, Tuberculosis

Mycobacterium avium subspecies *paratuberculosis* (MAP) causing Johne's disease (JD) is a major problem in animals having impact on health and productivity of domestic livestock world-wide. JD, though primarily a disease of ruminants, also infects pigs, dogs, horses, cat[1-4], rabbits, blue bulls, bison, deer, etc.[5-8], and non-human primates[9,10]. In US, more than 70% of dairy herds are infected with Bovine Johne's Disease (BJD), causing $200-250 million annual loss to dairy industry. In India too, BJD has been reported to be endemic causing high morbidity and reduced productivity. Recently, Vinodh Kumar[11] estimated the economic loss due to JD in India at Rs. 1,840 (US$ 38.33) per sheep/farmer/year. With 214.35 million (FAO, 2013) cattle population, India, ranks highest in the world. However, there has been a sharp rise in the number of low and unproductive cows, primarily due to BJD. As cow-slaughter is banned and cannot be salvaged for meat, farmers are shifting to buffaloes, goats and sheep husbandry. Unproductive cows are often let-off to roam free on roads. These cows are herded in *gaushalas*. Cows in *gaushalas* suffer from poor nutrition, unhygienic conditions and clinical BJD.

Serological detection of antibodies for MAP in domestic ruminants has been used in screening, sero-survey and diagnosis of MAP infection. Traditional, Agar gel precipitation test (AGPT) is also frequently used for the diagnosis of disease[12]. In the present study, AGPT using antigen from MAP culture (ATCC MAP strain 19698) is compared with 'indigenous' ELISA developed using antigen from MAP strain 'S5' (Indian Bison Type biotype) of goat origin for diagnosis of MAP infection in the cow naturally infected with Johne's disease.

Materials and Methods

History and collection of samples—Serum samples (160) were collected from healthy native milch cattle breeds, *Sahiwal* and *Hariana* of *Shri Mata Gaushala* at Barsana, Mathura, Uttar Pradesh, India and stored at −20 °C until screening. The stored samples were put to screening through two tests (ELISA and AGPT) independently for BJD.

Agar Gel Precipitation Test (AGPT)—AGPT was performed as per Woernle[13] using MAP strain (ATCC 19698) imported from UK for making Johnin to screen the cows. Briefly, 12 mL of 1.25% agar solution having 8% NaCl was dispensed in the

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sterilized petri dishes. After the solidification of agar, wells of 3 mm diameter with inter distance of 3 mm were made. In the central well, 30 µl of sonicated antigen of MAP (ATCC 19698) was poured and the test samples of serum were placed in the adjacent wells. Petri plates were incubated at 37 °C for 24 h and were examined against the dark background for precipitation lines.

Enzyme-linked Immuno-sorbent Assay (ELISA)—Indigenous ELISA kit initially developed for goats[14] and already standardized for screening cattle in India[15] was used. The antigen (soluble PPA) was prepared from the 'Indian Bison Type' bio-type of MAP strain 'S5', isolated from a *Jamunapari* breed of goat terminally sick with Johne's disease[16]. Antigen from 'Indian Bison Type' strain was standardized at 0.1 microgram/well of micro-titre plate. The test serum samples were diluted in ratio of 1:50 and anti-cattle horseradish peroxidase conjugate (Sigma Aldirch, USA) was used in 1:8000 dilution. Cattle serum samples collected from the animals which tested positive and negative in fecal culture were used as positive and negative controls, respectively. The optical densities (OD) values were transformed into sample-to-positive (S/P) ratios as per Collins method[17]. Samples in strong positive and positive category were considered positive for MAP infection.

Statistical Analysis—McNemar's test was applied to measure the significant difference between results of both tests[18,19].

Table 1—'Indigenous ELISA' and AGPT screening for MAP infection in cattle

Animals (n) / Breeds	Indigenous ELISA			AGPT
	Strong Pos (SP)	Positive (P)	Total Positive (SP+P)	Positive
160 *Sahiwal* and *Hariana*	3 (1.9)	78 (48.8)	81 (50.6)	66 (41.8)**

*Figures in parenthesis are percent

**Number of samples examined through AGPT= 158

Results and discussion

Out of 160 cows screened for MAP infection, 81 (50.6%) tested positive in 'Indigenous ELISA' and 66 (41.8%) in AGPT test. Of the total ELISA positive cows, only 1.9% were in strong positive category while 48.8% were positive (Table 1). Two tests together detected that 58.9% of the total cows were MAP infected (Table 2). Exclusively, 17.1% tested positive in ELISA, and 8.2% in AGPT. About 33.5% tested positive in both AGPT and ELISA. There was 74.7% agreement and 25.3% disagreement in the two tests. Screening of 92 AGPT negative cows by 'Indigenous ELISA' revealed the following: 7.6% negative; 30.4% suspected; 32.6% low positive; and 29.3% positive. Whereas, the 66 AGPT positive cows when put to ELISA, 3% proved negative; 10.6% suspected; 6.1% low positive; and 75.8% positive. About 4.5% were strong positive. Total number of cows tested positive/strong positive in both ELISA and AGPT (33.5%) were taken as true positive (TP). The cows (41.1%) which tested negative in AGPT and further proved negative, suspected and low positive when put to ELISA were declared true

Table 2—Comparative detection of MAP in cows by 'Indigenous ELISA' and AGPT

Tests	Status *n* (%)									
AGPT (*n*)=158	Negative – 92 (58.2)					Positive – 66 (41.8)				
	N	S	LP	P	SP	N	S	LP	P	SP
ELISA (*n*)=160	7 (7.6)	28 (30.4)	30 (32.6)	27 (29.3)	0 (0.0)	2 (3.0)	7 (10.6)	4 (6.1)	50 (75.8)	3 (4.5)
	TN 65 (70.7)			FN 27 (29.3)		FP 13 (19.7)			TP 53 (80.3)	

FN, false negative; FP, false positive; LP, low positive; N, negative; P, positive; S, suspected; SP, strong positive; TN, True negative; TP, True Positive

*Figures in parenthesis are percent

Total positive – 93 (58.9%);

AGPT – Positive (53+13) = 66 (41.87%) Negative = 92 (58.2%)

ELISA = Positive (53+27) = 80 (50.6%) Negative = 78 (49.4%)

Agreement: 118/158 = 74.6%; Disagreement: 40/158 = 25.3%

Screening: 158: TP 53 (33.5%) and TN 65 (41.1%); 158: FN 27 (17.1%); and FP 13 (8.2%)

negative (TN). The results of the two tests were found to be significantly ($P<0.05$) different from each other.

Johne's disease being chronic in nature is spectral disease, and hence infection is generally endemic in domestic livestock worldwide. Therefore, screening infected animals in a herd or flock at any given point of time come across different stages of infection, and thereby yield a gradient immune response. Animals in the active phase of infection (positives) and very active phase (super shedders) of disease can be identified using ELISA test on the basis of likelihood ratio[17]. However, in AGPT these differences are not quantitative but qualitative.

Generally, culture technique is not preferred in routine diagnosis of MAP infection due to long incubation period and high cost of mycobactin J. In the absence of culture, there is no 'Gold Standard' test for the diagnosis of MAP infection. ELISA is quick, easy to perform, cost effective, repeatable, sensitive and highly specific. Hence, we employed two serological tests (ELISA and AGPT) using two different antigens of MAP to replace culture as 'Gold standard'. Two antigens together detected 53 (33.5%) and 93 (58.9%) cows as true and total positives, respectively. Comparison of two tests showed that 'indigenous ELISA' using PPA (protoplasmic antigen) from native and novel isolate 'S5' of MAP ('Indian Bison Type') was more sensitive than AGPT using imported MAP strain (ATCC 19698) (Table 2). Poor sensitivity of AGPT was evident since some of the cows which showed low positive and negative by AGPT tested positive in ELISA. This further confirmed higher sensitivity of ELISA as compared to AGPT[20]. Higher sensitivity of ELISA test could be attributed to use of protoplasmic antigens from native strain ('S5') of MAP ('Indian Bison Type') in the indigenous ELISA while in AGPT the imported MAP strain ('cattle type') as mentioned above. However, AGPT assay showed higher specificity in clinical cases of disease but had lower sensitivity in early phase of disease[21]. Despite higher (>90%) specificity of AGPT in clinically infected animals it is not consistently used for diagnostic[22] purposes since sensitivity is low (30%). ELISA has been reported to be effective and efficacious compared to PCR, recommended as good supportive test for the diagnosis of MAP infection in camels[23]. Other studies using AGPT reported 38.7[24], 29.1[25] and 64.7%[26] of samples precipitating antibodies. Screening of positive and negative sera samples by AGPT using indigenous ELISA helped to find the infection level (gradient to correlate sero-titer with disease status) and also the true positives and true negatives. The cows those were found false negative (17.1%) and false positive (8.2%) where there was disagreement in two tests can be tested by microscopy or PCR.

The present study indicated that 'Indigenous ELISA' and AGPT using two antigens from different sources could be a better method for screening and diagnosis of MAP infection in herds and flocks. Individually, indigenous ELISA using antigen from native source of MAP proved superior to AGPT in the diagnosis of JD in cows.

Conflict of interest

The authors declare that they have no conflict of Interest.

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