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Seroepidemiology of paratuberculosis in cattle population of organized and unorganized farms of India

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ABSTRACT

Serum samples (531) collected randomly from cattle of organized (200) and unorganized (331) farms of Central and Northern parts of India were subjected to a commercial ELISA to know the seroprevalence of paratuberculosis. These serum samples were also tested by an in-house absorbed ELISA developed in the laboratory and the results were compared. The overall seroprevalence of paratuberculosis in cattle was 8.09% with significantly higher prevalence in cattle of organized farms (13.5%) than unorganized farms (4.83%). The seroprevalence was also significantly higher in calves (17.24%) than adults (7.57%); whereas there was no significant difference in prevalence rate among male (6.71%) and female (9.87%) cattle. Region wise the seroprevalence was slightly higher in organized farms of Northern India (16.43%) than that of Central India (11.81%). The sensitivity and specificity of in-house ELISA were 71.11% and 98.76%, respectively, with the accuracy of over 96%. On the basis of *Kappa*-test, the in-house ELISA was in good agreement with commercial Pourquier® ELISA and can be recommended for screening of paratuberculosis infection in cattle in India.

Key words: Cattle, ELISA, Paratuberculosis, Seroprevalence

Amongst various bacterial diseases affecting cattle health and production, paratuberculosis (Johne's disease) is considered as one of the economically important chronic diseases. The disease affects all domestic and wild ruminants, and is caused by *Mycobacterium avium* sub-species *paratuberculosis* (MAP). The economic losses are mainly due to reduced production, reduced feed efficiency, increased culling of infected animals and increased costs of testing procedure and control measures (Behr and Collins 2010). Paratuberculosis is worldwide in distribution and considered a threat to the dairy sector, beef/meat industry and livestock trade in many countries including India (Lombard 2011). The true incidence of JD is hard to estimate since the most infected cattle are asymptomatic, and there are difficulties in diagnosing cows with early infection (Behr and Collins 2010). Unknown status of the disease at organized farms as well as in the suburban and rural areas is a big obstacle in combating paratuberculosis in India. Amongst various diagnostic tests, ELISA is considered as an important serological test for screening of large number of animals on a herd basis (OIE 2014). ELISA provides rapid and cost-effective alternative diagnostic tool, and has the highest sensitivity amongst all serological tests (Singh *et al*. 2010). In the present study, sera from cattle of organized and unorganized farms of Central and Northern parts of India were tested by a commercial ELISA to know the incidence of paratuberculosis. These serum samples were also tested by an in-house absorbed ELISA developed in the laboratory and the results were compared with the commercial ELISA to know the comparative efficacy of in-house ELISA in the detection of MAP antibodies.

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MATERIALS AND METHODS

Animals and serum samples: The serum samples from 531 cattle were collected randomly from adults and calves of either sex from different organized and unorganized farms of Central (Nagpur, Akola and Durg) and Northern (Bareilly and Palampur) India, from 2008–09 (Table 1). The cattle on organized farms were raised under intensive management conditions. Among unorganized farms, the bullocks of slaughterhouse were brought from individual dairy farmers from nearby villages and slaughtered within the same day; whereas the cattle of Go Anusandhan Kendra were the stray animals kept for shelter.

Seroprevalence: The serum samples collected were subjected to a commercial Pourquier® ELISA kit as per the instructions of the manufacturer.

In-house ELISA (absorbed ELISA): Protoplasmic antigen (capture antigen) from MAP 316F strain and absorbing antigen from *Mycobacterium phlei* were prepared as described previously (Rajukumar *et al.* 2001, Tripathi *et al.* 2006). The optimum concentrations/dilutions for capture

and absorbing antigens, sera and conjugates were determined by checkerboard titration. The flat bottom 96-well plates were coated with capture antigen and incubated overnight at 4°C. All subsequent incubations and washings were carried out at 37°C and in phosphate-buffered saline containing 0.5% Tween-20 (PBST), respectively. Uncoated sites in the wells were blocked with 3% BSA in PBST at 37°C for 1 h. Sera were mixed with absorbing antigen and incubated at 37°C for 30 min and were added in duplicate wells. After addition of anti-bovine IgG-HRPO conjugate, colour was developed by addition of hydrogen peroxide and O-phenylenediamine. The optical density (OD) was measured at 492 nm in an ELISA reader. Known positive and negative serum samples were always included in each plate. The absorbance values for sera selected for testing were expressed and calculated in terms of sample to positive ratio (S/P values) using the following formula

$$\text{S/P value (\%)} = \frac{\text{sample } OD_{492nm} - N}{P-N} \times 100$$

P, known positive control mean; N, known negative control mean.

To determine the cut-off value for ELISA positivity, absorbance from sera of cattle that tested negative in bacterial culture, PCR and by commercial ELISA kit was used as negative control. Similarly, known positive cattle sera (12) available in the laboratory were also tested by both the ELISA. S/P value of ≥70% was considered the cut off for declaring positive results.

Comparison of results: The results obtained from in-house and commercial ELISA tests were statistically analyzed for various values, viz. sensitivity, specificity, predictive values for positive test, predictive values for negative test and kappa values as per Thrusfield (2005). The degree of association between each risk factor and the seroprevalence was also assessed by Pearson Chi-square ($\chi 2$) test using SPSS 16 statistical software.

RESULTS AND DISCUSSION

In the present study, a commercial ELISA was used to assess the seroprevalence of paratuberculosis in the selected organized and unorganized farms of Maharashtra, Chhattisgarh, Uttar Pradesh and Himachal Pradesh, as this ELISA was found to be sensitive and highly specific in ruminant population of India (Tripathi *et al.* 2007, Gupta *et al.* 2012).

The number of seropositive cases by Pourquier® ELISA in cattle of different organized and unorganized farms is given in Table 1. The overall seroprevalence of paratuberculosis in cattle (males and females) of organized and unorganized farm was 8.09% by Pourquier® ELISA. The prevalence of paratuberculosis in organized farms (13.5%) was significantly higher than that of unorganized farms (4.83%). Region wise the seroprevalence was slightly higher in organized farms of Northern India (16.43%) than that of Central India (11.81%) but the difference was not significant. Age-wise the seroprevalence of paratuberculosis was significantly higher in calves (17.24%) than adults (7.57%), whereas sex-wise there was no significant difference between the male (7.43%) and female (9.87%) cattle.

Out of 531 sera tested, 43 were positive and 488 were negative by Pourquier® ELISA. The in-house ELISA detected 36 positives and 495 negatives. Of these, 6 sera tested positive by the in-house ELISA but negative by Pourquier® ELISA, were considered 'false positive'. While 13 sera tested positive by Pourquier® ELISA, but negative by in-house ELISA, were considered 'false negative' (Table 2).

Table 2. Comparative results of in-house and commercial Pourquier® ELISA

Number of sera	Pourquier® ELISA	In-house ELISA	Both test
Total positive	43	36	32
Total negative	488	495	480
Total sera tested	531	531	-

True positive, 32; false positive, 6; false negative, 13; true negative, 480.

Table 1. Details of serum sample collection from cattle of organized and unorganized farms with number of seropositive cases by Pourquier® ELISA

S. No.	Farm location	Sex		Age	No. seropositive
		Male	Female		
	Organized farm				
1.	Cattle Farm, Akola, Maharashtra	-	30	Adult	4 (13.33%)
2.	Cattle Breeding Farm, Nagpur, Maharashtra	-	36	Adult	3 (8.33%)
3.	Bull Depot, Nagpur, Maharashtra	29	-	Calves	5 (17.24%)
4.	Cattle Breeding Farm, Durg, Chhattisgarh	-	32	Adult	3 (9.37%)
5.	Animal Disease Research and Investigation Farm (ADRIF), Bareilly, Uttar Pradesh (UP)	-	29	Adult	8 (27.58%)
6.	Baijnath Farm, Palampur, Himachal Pradesh	-	44	Adult	4 (9.09%)
	Unorganized farm				
1.	Slaughterhouse, Nagpur, Maharashtra	269	-	Adult	15 (4.53%)
2.	Go Anusandhan Kendra, Nagpur, Maharashtra	-	62	Adult	1 (0.3%)
Grand total = 531		298	233		43 (8.09%)

Both the tests revealed 32 sera as positive and were considered as "true positive" while 480 sera found negative in both the tests were considered as "true negative". The sensitivity, specificity, efficiency/accuracy, predictive value for positive test and predictive value for negative test were 71.11, 98.76, 96.42, 84.21 and 97.36%, respectively. *Kappa* statistics was used to know the agreement between tests by calculating following parameters.

(i) Observed proportion agreement between the two tests (OP) = 0.96; (ii) Expected proportion of agreement by chance (both positive) (EP+) = 0.006064; (iii) Expected proportion of agreement by chance (both negative) (EP-) = 0.85; (iv) Expected proportion of agreement by chance (EP) = 0.8560; (v) Observed agreement beyond chance (OA) = 0.104; (vi) Maximum possible agreement beyond chance (MA) = 0.144; (vi) *Kappa* (ratio of the OA/MA) = 0.72.

On the basis of *Kappa* value (0.72) calculated in this study to know agreement between the two tests, the in-house ELISA used in this study was found to be in good agreement with commercial Pourquier® ELISA.

The estimation of prevalence of MAP infection is required for taking appropriate measures to control the disease. The ELISA methods used in diagnosis of bovine paratuberculosis are rapid, cost-effective and characterized in general by their high sensitivity and specificity (Tripathi *et al.* 2007, Singh *et al.* 2010). Therefore, it is considered as an important test for screening of large number of animals on a herd basis. The overall seroprevalence of paratuberculosis reported in organized cattle farms of the present study is comparable with previous reports from different parts of India such as 15.14% in Bengaluru (Karnataka) (Gupta *et al.* 2012), 7% in Uttar Pradesh (Trangadia *et al.* 2014), 15.6% in Punjab (Garg *et al.* 2015) and 10.83% in Andhra Pradesh (Didugu *et al.* 2015). However, other studies reported the prevalence as high as 26.9 to 50.6% in cattle farms of North India by ELISA (Singh *et al.* 2010, Kumar *et al.* 2014, Pahangchopi *et al.* 2014). Among organized farms of the present study, highest prevalence of paratuberculosis was recorded in the cattle population of ADRIF (Animal Disease Research and Investigation Farm), Bareilly (Uttar Pradesh). This was expected since these cattle were kept in separate herds due to history of prolonged emaciation, weakness and lack of production. The significantly lowered MAP antibody prevalence in cattle of unorganized sectors as compared to organized farms may be due to management and animal health practices, where the possibility of introduction of infected cattle from an organized farm is generally rare. Also animals in unorganized farms are usually not continuously exposed to infected animals in comparison to organized farms, where animals live in the highly contaminated environment. Environmental factors and farm level management practices are also associated with the incidence and occurrence of disease in farm and farmers herds (Garg *et al.* 2016). Recently multivariate analysis showed contamination of feed and water with adult manure and history of chronic diarrhoea in the herd as the factors significantly associated with positive status of animals in the herd (Garg *et al.* 2016).

Interestingly, the seroprevalence rate was significantly higher in calves than adults. This is in agreement with the observation that there is an age dependent increase in resistance to MAP, and animals are usually infected as calves with clinical signs appearing later in life (Mortier *et al.* 2013). Since these calves were maintained in a separate herd from their dams after 6 months of age, it may be possible that they acquired MAP infection early in life from contaminated herd environment. Dieguez *et al.* (2008) also reported that calves housed with adults before 6 months of age in the herd were found more prone to MAP infection. The significantly higher prevalence rate in calves suggested that the control in cattle herds requires both herd management changes to limit faecal-oral infection spread and diagnostic testing to identify infectious cattle for segregation or removal (Kennedy and Benedictus 2001). Similarly, control programs should also emphasize more on prevention of transmission of infection, especially to susceptible young stock (Garg *et al.* 2016). Considering the physiological and stressor differences, it is postulated that clinical and pathological features of JD are different in bullocks than dairy cattle (Narnaware *et al.* 2016). However, no significant difference was recorded in the occurrence of the disease among male and female cattle of this study.

Control and management of Johne's disease are complicated by the lack of a rapid, sensitive diagnostic test for identifying diseased animals before clinical signs develop (Gupta *et al.* 2012). Sensitivity and specificity are of principal concern for any test when used for the detection of MAP infection. The absorption of bovine sera using a cellular extract of *M. phlei* prior to testing was important for high specificity of in-house ELISA used in the present study. In previous comparison studies of various commercial ELISAs and in-house ELISA in cattle, the sensitivity and specificity of in-house ELISA were reported to vary from 47.5–56.3% and 86.8-99%, respectively, which is comparable to the present study (Ferreira *et al.* 2002, Shin *et al.* 2008).

In conclusion, the indigenous ELISA of this study was in good agreement with commercial Pourquier® ELISA and hence can be recommended for screening of MAP infection in cattle in India. The considerable seroprevalence of MAP infection at different organized farms in India as observed in this study and those reported earlier suggested that efforts are needed for the control of paratuberculosis in India.

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