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Sero-Surveillance of *Mycobacterium avium* subspecies *paratuberculosis* Infection in Domestic Livestock in North India Using Indigenous Absorbed ELISA Test

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Abstract: A total of 829 serum samples belonging to domestic livestock (Cattle, buffaloes, goat and sheep) and driven from different parts of North India between 2005 to 2008, were screened to estimate the seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection using 'indigenous absorbed ELISA kit'. Seroprevalence of MAP in the domestic livestock was 23.1%. Prevalence was higher in large ruminants (24.1%) as compared to small ruminants (22.5%). Highest seropositivity was in cattle (26.9%), followed by goats (23.9%), buffaloes (20.2%), and sheep (19.0%). In cattle region-wise, 25.8, 29.1 and 30.7% animals were positive from Mathura (UP), Rohtak (Haryana), and Bareilly (UP) regions, respectively. In buffaloes, the highest prevalence was found at Bareilly (26.6%) followed by Rohtak (20.0%) and Baghpat (18.4%) regions. In goats, 19.6, 37.5, 40.0 and 21.9% animals were positive from Mathura (farm herd), Etawah, Agra and Ajmer (farmers herd) regions, respectively. In sheep, prevalence of MAP was 25.5 and 16.3% in Mathura and Mannavanur regions, respectively. In sheep, prevalence was higher in Northern region as compared to the Southern region of the country. The present study showed that the prevalence of MAP in domestic livestock was moderately higher; therefore there is an urgent need to control the disease at National level in order to improve per animal productivity in the country.

Keywords: Johne's disease, *Paratuberculosis*, *Mycobacterium avium* subspecies *paratuberculosis*, Seroprevalence, ELISA.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), the cause of Johne's disease (JD) or Paratuberculosis in animals is incurable, chronic wasting and debilitating disease characterized by weight loss and profuse diarrhea (Whitlock and Buergelt, 1996). Though JD is principally a disease of ruminants, MAP can also infect non-ruminant animal species (pigs, dogs, horses, cat etc.) including free ranging animals (Blue bull, deer, rabbits) and primates (Chiodini *et al.*, 1984). JD adversely affects animal productivity leading to huge economic losses and has been recognized as one of the most costly infectious diseases of dairy cattle in the world (Hasonova and Pavlik, 2006). The morbidity rates are very high and distributed over a period of time, thus losses in production go unnoticed and have never been estimated in India despite low per animal productivity (Barbaruah and Joshep, 2008). A countrywide survey of MAP infection has not been undertaken so far, though JD is endemic in domestic livestock (Singh *et al.*, 2008a; Sharma *et al.*, 2008; Kumar *et al*., 2007). Country at present lacks any commercial indigenous, cost-effective and sensitive diagnostic kits. Culture though 'Gold standard test' for JD (Rideout *et al.*, 2003), due to high cost, low sensitivity and the long incubation period, has limited use. PCR cannot distinguish between dead pass-through bacilli or those arising from colonization of the intestines. Serology provides rapid and cost-effective alternative diagnostic tool and ELISA has high sensitivity in serological tests. Previous studies

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compared native semi-purified antigen (NSPA) of MAP prepared from a native strain of MAP of 'Indian Bison type' genotype with commercial purified protoplasmic antigen (CPPA) of Allied Monitor Inc., (US) of 'cattle type' genotype in indirect (unabsorbed) ELISA kit and commercial (absorbed) ELISA kit for the screening of goats and sheep against MAP infection. This study reported better performance of 'indigenous ELISA kit' (Singh *et al.,* 2007). Commercial ELISA kit showed lowered sensitivity and higher specificity in comparison to 'Indigenous ELISA kit' (Singh *et al.,* 2007). In the present study 'indigenous ELISA kit' initially developed for the screening of goat and sheep (Singh *et al.,* 2007) was modified (Singh *et al.,* 2009) and used for the screening of domestic livestock in north India.

2. Materials and Methods

2.1 Serum samples

A total of 829 serum samples were collected from different parts of North India between 2005 and 2008 and were screened at Microbiology laboratory of Central Institute for Research on Goats (CIRG), Makhdoom, (UP). Of the 829 serum samples, 193, 143, 351 and 98 belonged to cattle, buffaloes, goats and sheep, respectively. Serum samples were collected aseptically, transported to the laboratory under ice and kept without adding any preservative at -20^{0}C until used.

2.2 Control (negative and positive serum) samples

Serum samples collected from suspected, extremely weak cattle with known positive status for MAP in fecal culture was used as positive control. Serum samples from healthy cattle belonging to the farmer's herd, with known culture-negative status for MAP infection was used as negative control for the screening of cattle and buffaloes serum samples. However, for goat and sheep positive and negative serum controls were selected from goats positive and negative in culture in earlier studies (Singh *et al.,* 2009).

2.3 Modified 'Indigenous absorbed ELISA kit'

'Indigenous ELISA kit' was used for the screening of MAP infection in domestic animals (Singh *et al.,* 2008) was modified to improve the specificity by adding absorption step using *M. phlei* culture (Allied Monitor Inc., USA), in the protocol (Singh *et al.,* 2009) and was used in the present study for the screening of domestic livestock.

2.4 Test proper

The 0.1µg of sonicated protoplasmic antigen in 100µl of carbonate-bicarbonate buffer (pH 9.6) was used for coating in duplicate wells of flat bottom 96 well ELISA plate (Greiner Bio-One). The plates were kept for overnight at 4^{0}C followed by washing thrice with washing buffer PBST (PBS with 0.05% Tween 20). Blocking was done by 200µl of 3% skimmed milk in PBS and incubated at 37^{0}C for one hour. After incubation plates were washed thrice with PBST, after washing the plates, 100µl of 1:50 diluted test serum samples in dilution buffer (PBST containing 1% BSA and 2mg/ml *M. phlei*) were added to each well, in the duplicates and incubated for 2 hours at 37^{0}C. After incubation three washings (5 minutes each) were given with of PBST. Then 100µl of optimally diluted (1:8000) conjugate (Sigma) in PBS was added in all and incubated for an hour at 37^{0}C. After incubation, the plate was washed three times with PBST. Finally, 200µl of freshly prepared substrate (orthophenylene diamine dihydrochloride-OPD), 5mg per plate in substrate buffer (pH 5.0) was added to each well. Following incubation (in the dark) for 20 minutes at room temperature, absorbance was read at 450nm in ELISA reader (Multiscan, Lab system, USA) without adding of stop solution (5N H_2SO_4). Blank, positive and conjugate controls were also run along with serum sample on each plate.

2.5 Calculation of cutoff

Cutoff value was calculated as per the method of Collins (2002). Serum samples were run in duplicate and mean OD was taken as the final OD value. OD values of the test samples were transformed to S/P ratio and animals in the strong positive category were considered positive for infection with MAP.

3. Results and Discussion

In the present study, using 'indigenous absorbed ELISA' kit, seroprevalence of MAP in domestic animals of North India was 22.1% (Table 1). A previous study from north India reported slightly high (29.0%) prevalence of MAP in large ruminant using indigenous unabsorbed ELISA kit as the diagnostic test (Singh *et al.,* 2008a). Lower prevalence of MAP in the present study may be due to the use of absorption of serum with *M. phlei*, which minimize the cross-reaction of MAP with other mycobacteria (Yokomizo *et al.,* 1983). Though, cross-reactivity is the major problem for the diagnosis of mycobacterial infections (Yokomizo *et al.,* 1983). Species-wise 21.7, 16.0, 26.2 and 19.0% samples were positive for MAP from cattle, buffalo, goat and sheep, respectively. Prevalence of MAP was higher in large ruminant (cattle and buffalo) in comparison to small ruminant (Goat and sheep). Higher prevalence of MAP in large ruminant may be attributed to the high endemicity of the MAP infection and other production stress factors. Prevalence of MAP was higher in cattle (21.7%) as compared to buffaloes (16.0%) in north India. This finding indicated that the unproductivity of cattle in India may be to the JD.

Similar to the present study, Singh *et al.*, (2008a), reported, 29.8 and 28.6% animals were positive from cattle and buffaloes in North India using an unobserved ELISA test. Sivakumar *et al.*, (2005) screened the cattle sera from dairy and organized farm from Chennai, 21.3% were positive in ELISA, 10.8% of these ELISA positive animals were confirmed by AGID. In another study, using ELISA kits in serum samples of Buffaloes gave 46.7% seroprevalence (48.0% by target tissue culture) in farmer's buffaloes sacrificed for meat purpose in the Agra region (Yadav *et al.*, 2008). Siva Kumar *et al.*, (2005) reported 22% and a 10% incidence of MAP in cattle and buffaloes of Bareilly region.

As seen in large ruminants, higher prevalence of MAP infection was found in small ruminants (goat and sheep). Prevalence of MAP was higher in goat (23.9%) as compared to sheep (19.0%). Higher prevalence of MAP in goats may be due to the high population density and endemicity of MAP in goat herds (Singh *et al.*, (1996); Singh *et al.*, (2008a); Vohra *et al.*, (2008). Similarly, Singh *et al.*, (1996) also reported higher prevalence of MAP in goats as compared to sheep using microscopy and fecal culture. Recently, Singh *et al.*, (2008b) screened the 35 goats from the Agra region by fecal culture and ELISA test, 77.1 and 40.0% goats were positive for MAP infection, respectively. Of the 40% ELISA positive goats, 71.4% were also positive in fecal culture. Previous studies also reported higher prevalence of MAP in farm herds as compared to farmer's herds (Kumar *et al.*, 2007). However, in the present study, no major difference was found in the prevalence of MAP in farm and farmer's herds. This may be due to the sharing of domestic livestock by the farm and farmer's herds in north India, confinement of animals due to lack of pasture land and free grazing resources.

Table 1. Screening of domestic livestock against MAP infection by indigenous ELISA test.

Ruminant	Species	Region	Management	Sample	Positives	Positives %
Large Ruminant	Cattle	Mathura	Farm	143	37	25.8
		Rohtak, Haryana	Farmer	24	7	29.1
		Bareilly	Farm	26	8	30.7
		Total		193	52	26.9
	Buffalo	Baghpat	Farm	38	7	18.4
		Rohtak, Haryana	Farmer	90	18	20.0
		Bareilly	Farm	15	4	26.6
		Total		143	29	20.2
	Subtotal			336	81	24.1
Small Ruminant	Goat	CIRG, Mathura	Farm	163	32	19.6
		Etawah	Farm	80	22	37.5
		Agra	Farmer	35	14	40.0
		Ajmer	Farmer	73	16	21.9
		Total		351	84	23.9
	Sheep	CIRG, Mathura	Farm	43	11	25.5
		Mannavanur	Farm	98	16	16.3
		Total		142	27	19.0
	Subtotal			493	111	22.5
Grand total				**829**	**192**	**23.1**

4. Conclusion

High prevalence of MAP infection in farm and farmer's herds of domestic ruminants in North India is directly related to the low per animal productivity and there is increased risk of exposure of human beings to MAP infection. Therefore, it is necessary to control the MAP infection in animals first in order to safeguard human population. National JD control programs are essential to reduce the prevalence of MAP in India and to improve per animal productivity and also to reduce the risk of exposure to human beings.

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