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RESEARCH ARTICLE

BIO-BURDEN AND BIO-TYPE PROFILES OF *Mycobacterium avium* SUBSPECIES *paratuberculosis* INFECTION IN SUSPECTED POPULATION OF DOMESTIC LIVESTOCK IN INDIA

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ABSTRACT

Multiple diagnostic tests standardized in the laboratory are central to diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), cause of Johne's disease (JD) in domestic livestock, Information on bio-burden of MAP infection at animal, species, region, state and National levels is limited in India. In absence of National prevalence estimates (bio-burden), the disease has not received due attention and priority for the control. Present study estimated bio-burden of MAP in domestic livestock from different agro-climatic regions of country. Clinical samples (faeces, blood and serum) of suspected animals from farm and farmer's herds / flocks, submitted to CIRG, Makhdoom between 2010 and 2011, were screened using multiple diagnostic tests (microscopy and IS900 PCR on faecal blood and Indigenous ELISA on serum samples). Of the 716 faecal samples screened by microscopy, 50.9% were positive for acid-fast bacilli like MAP. of the faecal (n=183) and blood (n=510) samples screened using IS900 PCR; 27.3 and 19.6% were detected positive, respectively. Screening of 1598 serum samples (goats, sheep and cattle), by 'Indigenous ELISA kit' 68.4% were positive. The study reports very high bio-burden of MAP in domestic livestock population. Molecular characterization of MAP using IS*1311* PCR-REA identified 'Bison type' as predominant biotype (98.6%) in domestic livestock population.

INTRODUCTION

Mycobacterium avium subspecies paratuberculosis (MAP), the cause of Johne's disease (JD) or Paratuberculosis in domestic livestock, is responsible for huge economic losses in domestic livestock productivity worldwide. JD, though primarily a disease of domestic ruminants but has also been associated as principal etiology of inflammatory bowel disease or Crohn's disease in human beings (Greenstein, 2003). India possesses huge population of domestic ruminants [goats (154 million), sheep (73 million), cattle (210 million) and buffaloes (111 million)] (FAO, 2012). Though India is leading milk producer (117 million tonnes) in the world, but these achievements are number driven and per animal productivity is much below the world averages and is 1/6 in Asian countries. Despite low per animal productivity, there is no attempt to study National prevalence of Johne's disease in domestic livestock. Similar situation exists in most of the world outside developed countries. This is mainly due to lack of field based indigenous test, poor sensitivity and specificity of Johnin test, poor sanitary conditions, lack of priority and control measures, JD continues to spread vertically and horizontally in animal and human population. Previous limited surveys reported JD as one of the major infectious disease of domestic livestock (Singh *et al*., 2008).

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In the absence of prevalence estimates JD has never been priority for the control. Present study diagnosed MAP infection in clinical samples (faecal, blood, serum) submitted to our laboratory (Animal Health Division of Central Institute for Research on Goats, Makhdoom) mainly from suspected population in a calendar year (2010 to 2011). IS*1311* PCR-REA was employed to bio-type MAP strains infecting domestic livestock. Present findings are expected to bring JD in focus and may create environment for Nation-wide debate for the need of National estimates on prevalence of JD and initiation of control programs in India, which may serve as model for other developing and poor countries of the world where status of MAP infection is not known either in animals or in human beings.

MATERIALS AND METHODS

Animals and Clinical Samples

Our laboratory is one of the very few facilities in the country which has been working on paratuberculosis research (diagnosis and control) continuously since 1984. Samples from different livestock species and parts of the country are regularly submitted mainly for diagnosis. However, majority of these samples come from suspected population of domestic livestock. We have standardized 4 diagnostic tests (fecal microscopy, culture, IS*900* PCR and *1311* PCR-REA) on multiple samples (feces, blood, serum, milk and tissues). In view of the inherent difficulties and limitations of sampling, one time sampling, non-availability of proper, adequate and repeated clinical

samples, non-availability 100% specific and sensitive tests, cost of imported kits, frequent changes in laboratory man power etc., all adds to the woes of working with chronic infection like JD. These problems are only tip of the ice berg, and is main reasons, why very few researchers in resource poor countries like India enter in to the paratuberculosis research, Though we request for feces, blood and serum from each to the animal for confirming the diagnosis, however, it is almost impossible to get perfect sampling due to various reasons in our social structure, disease management system, which is totally voluntary in developing and under developed countries. Due to limited laboratories, non-availability of OIE referral laboratory on JD in South Asia, working with indigenous kits, high cost of imported kits and reagents, lack of confidence among researchers on each other, in-appropriate sampling, and lack of information and will to know production losses etc., add to the complexities with respect to Paratuberculosis prevalence, diagnosis, bio-typing, control measures in countries outside developed nations, including India.

Present study analyzes, information on the bio-burden of JD based on the samples (feces, blood, serum and tissues) submitted from suspected population of domestic livestock different regions of the country (Northern states: Himachal Pradesh & Uttar Pradesh), Southern states (Kerala & Tamil Nadu), Western states (Gujarat & Rajasthan) and Central state (Madhya Pradesh), between April 2010 to March 2011. A total of 716 faecal samples (281 goats, 123 sheep and 312 cattle) were screened by fecal microscopy. Using 'Indigenous ELISA' kit a total of 1598 serum samples (885 goats, 141 sheep and 572 cattle) submitted were screened for the presence of MAP infection. IS*900* PCR was performed on 183 faecal samples (34 goats, 7 sheep, 85 cattle and 57 buffaloes) to confirm the MAP infection and 510 blood samples (105 goats, 141 sheep and 264 cattle).were analyzed for genotype of MAP (Tables 1, 2, 3, 4 and 5).

Microscopy

Approximately, 2.0 grams of fecal material was finely grounded in sterilized pestle and mortar with sterilized distilled water (10-12 ml) and centrifuged at 4500 rpm for 45 min at room temperature (RT). After discarding supernatant, middle layer was removed by sterilized swab and decontaminated in 0.9% HPC for 16-18 hrs. Smear was prepared from portions of sediment, stained by Ziehl Neelsen (ZN) staining (Singh *et al*., 2011) and examined for the presence of acid-fast bacilli (AFB) indistinguishable to *Mycobacterium avium* subspecies *paratuberculosis*.

IS *900* PCR

DNA was isolated from remaining decontaminated faecal material by methodology optimized previously in our laboratory (Kumar *et al*, 2007). DNA was also isolated from blood ('blood PCR') using our protocol (Singh *et al*., 2010b). Isolated DNA (fecal and blood) was subjected to IS*900* PCR as per Singh *et al.* (2010b), Briefly, PCR was set in volume of 50 µl, using 1.0-5.0 ng template DNA, 5 µl of 10X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µl of each primer (10 pmole) and 5 U Taq polymerase. Total of 37 cycles were performed in a thermo-cycler (MJ research) for complete amplification reaction. Thermal cycling conditions were: initial denaturation at 94^0C for 3 min, followed by 37 cycles of denaturation at 94^0C for 30 sec, annealing at 64^0C for 30sec, extension at 72^0C for 1 min, and final extension at 72^0C for 7 min. Presence and yield of specific PCR product (413 bp) was analysed by agarose gel electrophoresis.

IS *1311* PCR

Samples positive in IS*900* PCR were processed for IS*1311* PCR using M56 and M119 primers (Sevilla *et al*., 2005). Cyclic conditions were same as for IS*900* PCR except that annealing temperature of 62^0C was used. A product size of 608 bp was considered positive after separation on 2% agarose gel stained with ethidium bromide.

IS *1311* PCR-REA

IS*1311* PCR_REA was carried out as per Sevilla *et al*. (2005). Briefly, the reaction was carried out in a volume of 30 µl, containing 20 µl positive IS*1311* PCR product, 5 µl 10X buffer (Fermentas), and 2 U of each endonuclease *Hinf*I and *Mse*I (Fermentas). The reaction mixture was incubated at 37^0C for 2 hrs. Band patterns were visualised after electrophoresis on 4% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted as per Whittington *et al*. (2001).

Serum ELISA

ELISA was carried out using indigenously developed kit utilizing antigen from native strain. ELISA was carried out using indigenously optimized protocol (Singh *et al.*, 2009a) and results of ELISA were analyzed as S/P ratio as per method of Collins (2002). Samples in strong positive and positive category were taken as positive for MAP infection. Indigenous ELISA kit has shown comparable specificity and was highly sensitive, when compared with imported commercial kits (Singh *et al*., 2009b).

RESULTS

Faecal microscopy

Of 716 faecal samples screened using ZN staining, 365 (50.9%) were positive for acid fast bacilli (AFB). Of the 365 positive animals, 116 (31.7%), 55 (15.0%) and 191 (52.3%) were from goats, sheep and cattle, respectively. On the basis of samples submitted, state-wise bio-burden of AFB in feces of suspected animals was highest in Himanchal Pradesh (100.0%) followed by Rajasthan (90.9%), Madhya Pradesh (85.7%), Kerala (63.6%), Tamil Nadu (39.4%), Gujarat (36.7%), Uttar Pradesh (36.2%) and Assam (11.1%) (Table 1).

Faecal and blood PCR

Of 183 faecal and 510 blood samples screened directly by IS*900* PCR, 50 (27.3%) and 100 (19.6%) were positive for MAP infection, respectively (Figure 1). Species-wise, bio-burden of MAP by direct IS*900* PCR was highest in cattle (29.4%) followed sheep (11.6%) and goats (7.6%). State-wise bio-burden of MAP was highest (25.8%) in Uttar Pradesh as compared to Tamil Nadu (14.6%), Rajasthan (8.5%) and Gujarat (2.0%) using IS*900* blood PCR. Whereas, using IS*900* faecal PCR, bio-burden was highest in Himanchal Pradesh (85.7%), followed by Madhya Pradesh (50.0%), Tamil Nadu (25.0), Assam (25.0%), Uttar Pradesh (23.0%) and Rajasthan (14.3%) (Tables 2 and 3).

'Indigenous ELISA kit'

Of 1598 serum samples screened using 'Indigenous ELISA kit', 1093 (68.4%) were positive for MAP infection. Sero-positivity or bio-burden was highest in goats (81.1%), followed by cattle (57.5%), and sheep (32.6%). Region-wise, 100.0, 96.7, 91.2, 88.2, 84.4, 75.6, and 57.8% goats were positive from states of Himachal Pradesh, Kerala, Uttar Pradesh, Rajasthan, Punjab, Gujarat and Assam, respectively. In sheep, highest positivity was in Uttar Pradesh (92.9%) followed by Tamil Nadu (30.7%) and Rajasthan (7.7%). In cattle, 65.6 and 46.0% were positive from Uttar Pradesh and Gujarat, respectively (Table 4).

IS*1311* PCR-REA

All the IS*900* PCR positive samples were also positive in IS*1311* PCR. Of 150 samples screened, 7 could not be typed due to insufficient PCR product. 'Bison type' biotype was identified from 98.6% animals, whereas 2 (1.4%) samples were Cattle type' (Table 5, Fig. 2). Of 31 goats and 19 sheep positive samples, all were infected with 'Bison type' whereas majority of cattle (89 or 97.8%) were

Table 1. Prevalence of MAP on the basis of general screening of domestic livestock from different states by fecal microscopy (2010-2011)

S.No	State	Place	Species	Sample *(n)*	Positive *(n)*	%
1	Uttar Pradesh	Mathura	Cattle	179	101	56.4
			Goats	97	62	63.9
		Raibareily	Goats	6	6	100
		Agra	Cattle	85	64	75.3
2	Tamil Nadu	Mannavanur	Sheep	101	35	34.6
			Cattle	5	5	100
			Buffalo	3	3	100
3	Gujarat	Dantiwada	Cattle	37	18	48.6
			Goats	50	14	28.0
4	Assam	Guwahati	Goats	99	11	11.1
5	Madhya Pradesh	Bhopal	Goats	14	12	85.7
6	Rajasthan	Bikaner	Sheep	22	20	90.9
7	Himachal Pradesh	Palampur	Goats	4	4	100
			Cattle	3	3	100
8	Kerala	Trichur	Goats	11	7	63.6
Grand Total				**716**	**365**	**50.9**

Table 2. State-wise prevalence of MAP using fecal IS *900* PCR (2010-2011)

S.No	State	Place	Species	Sample *(n)*	Positive *(n)*	%
1	Uttar Pradesh	Mathura	Cattle	77	10	13.0
			Buffaloes	54	20	37.0
		Raibareilly	Goats	4	1	25.0*
		Total		**135**	**31**	**22.9**
2	Tamil Nadu	Mannavanur	Cattle	5	0	0.0
			Buffaloes	3	2	66.7*
		Total		**8**	**2**	**25.0**
3	Himachal Pradesh	Palampur	Cattle	3	2	66.7*
			Goats	4	4	100.0*
		Total		**7**	**6**	**85.7**
4	Assam	Guwahati	Goats	12	3	25.0
5	Madhya Pradesh	Bhopal	Goats	14	7	50.0
6	Rajasthan	Bikaner	Sheep	7	1	14.3
		Grand Total		**183**	**50**	**27.3**

*On the basis of suspected animal screening

Table 3. State-wise prevalence of MAP using Blood IS*900* PCR (2010-2011)

S.No	State	Place	Species	Sample ***(n)***	Positive ***(n)***	%
1	Uttar Pradesh	Mathura	Cattle	96	32	33.3
			Goats	54	7	13.0
		Agra	Cattle	168	43	25.5
		Total		**318**	**82**	**25.8**
2	Tamil Nadu	Mannavanur	Sheep	82	12	14.6
		Total		**82**	**12**	**14.6**
3	Rajasthan	Bikaner	Sheep	59	5	8.5
4	Gujarat	Dantiwada	Goats	51	1	2.0
			Grand Total	**510**	**100**	**19.6**

***** Positive % in goat 8 (7.6); sheep 17 (11.6); cattle 75 (29.4)

Table 4. State-wise prevalence of MAP using serum ELISA (2010-2011)

S.No	State	Place	Species	Sample ***(n)***	Positive ***(n)***	%
1	Uttar Pradesh	Mathura	Cattle	200	139	69.5
			Goats	70	62	88.6
			Sheep	14	13	92.9
		Agra	Cattle	135	81	60.0
		Bundelkhand	Goats	21	21	100.0*
		Total		**440**	**316**	**71.8**
2	Rajasthan	Bikaner	Goats	279	246	88.2
			Sheep	26	2	7.7
		Total		**305**	**248**	**81.3**
3	Gujarat	Dantiwada	Cattle	237	109	46.0
			Goats	299	226	75.6
		Total		**536**	**335**	**62.5**
4	Punjab	Ludhiana	Goats	90	76	84.4
5	Himachal Pradesh	Palampur	Goats	6	6	100.0*
6	Assam	Guwahati	Goats	90	52	57.8
7	Kerala	Trichur	Goats	30	29	96.7*
8	Tamil Nadu	Mannavanur	Sheep	101	31	30.7
		Grand Total		**1598**	**1093**	**68.4**

** Serum samples in 'strong positive' and 'positive' category in ELISA were considered as positive;
* On the basis of suspected animal screening.

Table 5. State-wise genotype profile of MAP using IS*1311* PCR-REA in IS*900* positive samples

S.No	State	Place	Species	IS900 positive DNA *(n)*	MAP Genotype	
					Indian Bison Type	Cattle Type
1	Uttar Pradesh	Mathura,	Cattle	85	83	2
		Agra,	Goats	17	17	0
		Raibareily	Sheep	4	4	0
		Total		**106**	**104**	**2**
2	Himachal Pradesh	Palampur	Cattle	6	6	0
			Goats	4	4	0
		Total		**10**	**10**	**0**
3	Tamil Nadu	Mannavanur	Buffaloes	2	2	0
			Sheep	15	15	0
		Total		**17**	**17**	**0**
4	Madhya Pradesh	Bhopal	Goats	7	7	0
5	Assam	Guwahati	Goats	3	3	0
			Grand Total	**143**	**141 (98.6%)**	**2 (1.4%)**

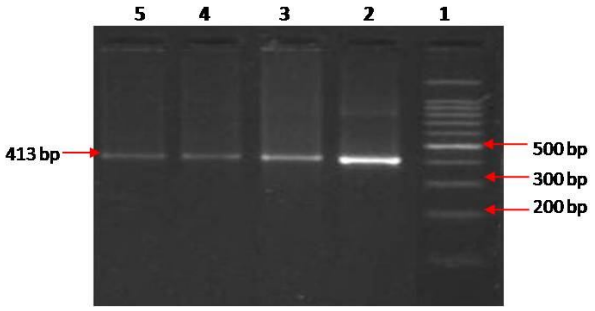

Fig 1. MAP specific amplicons (413bp) by PCR using IS*900* specific primers.
Lane 1: 100bp ladder, lane 2: Positive control (MAP DNA), lane 3-5: DNA samples isolated from blood

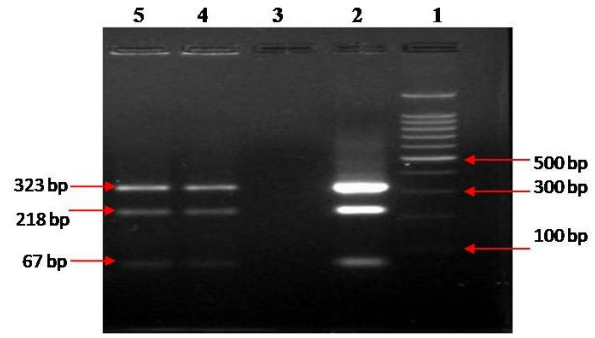

Fig 2. *IS 1311* PCR-REA analysis of *IS900* positive MAP DNA.
Lane 1: 100bp DNA ladder, lane 2: Positive control DNA ('Indian Bison Type'), lane 3: Negative control, lane 4 and 5: Digested DNA sample ('Indian Bison Type')

infected with 'Bison type' REA pattern, rest 2 (2.2%) were 'Cattle type' biotypes. In buffaloes all were 'Bison type'. 'Bison type' was most prevalent biotype in different domestic livestock species located in different states of the country.

DISCUSSION

Present findings are based on the samples from suspected domestic livestock submitted to Microbiology laboratory from different parts of the country in a calendar year (2010 to 2011). Though many of the samples (blood, serum and faeces) were from same suspected animals. Like many other developing and un-developed countries, in India also, sampling of desired animals based on random table number is one of the biggest constrains. Therefore, information on the National prevalence of livestock diseases and losses caused by disease, as available in developed countries, is extremely limited. Therefore genuine sampling in Indian conditions is extremely difficult specially from large ruminants in villages, where proper restraining devices are usually not available. Therefore, sampling in these conditions of cattle and buffaloes is usually based on convenience sampling (feces and milk, if in milking) or from the very limited suspected animals essentially needed to be screened. In these conditions it is not easy to present easy picture of diagnosis specially in chronic infections like JD, where, clinical symptoms are also vague and there is need of multiple samples (blood, serum, feces) and repeatedly from individual animal. In absence of frank clinical symptoms (sub-clinical JD), it is very difficult to convince owner that disease exists and there is need for screening or providing treatment to sub-clinically infected animals. However, in small ruminants like goats and sheep, collection of serum and feces is most convenient as compared to un-coagulated blood samples (Blood sample in EDTA). In the present study, fecal samples collection and submission was most convenient sample for large and small ruminants. Since in the present study mostly involves goats and sheep, therefore serum was most convenient sample, therefore we received maximum number of serum samples (1598), after that it was fecal (716) and un-coagulated blood was least (510). This is why the analysis of results was done sample-wise, animal-wise and state-wise, to arrive at best possible results under the prevailing limitations. In Indian conditions, cost of diagnosis is again a biggest constrain especially in case of goats and sheep, which have been traditionally reared as 'Zero in-put Agriculture'.

Cost-wise microscopy is the cheapest and PCR is costliest, whereas ELISA comes in middle. Similarly as per the convenience of processing again microscopy is the best. For every test (traditional or modern) it is human resource which plays vital role as compared to test itself, in countries which cannot afford ready to use and user friendly costly diagnostic kits on large number of samples. Therefore in the present study, fecal microscopy and indigenous serum ELISA kits were used as screening tests and IS*900* or IS*1311* PCR, to confirm MAP infection and to know bio-type of MAP, on limited number of samples, depending on source, species, origin and need on species and place of origin of sample. Present study report high bio-burden of MAP in the suspected population of domestic ruminants belonging to different agro-climatic regions of the country. Higher bio-burden of MAP is directly correlated with low per animal productivity and increased risk of human exposure. As reported in previous studies (Singh *et al*., 2009b; Singh *et al*., 2010a), 'Bison type' was the predominant biotype infecting domestic animals in different regions of the country. High bio-burden of MAP (species wise and state wise) reported in present study may be due to high endemicity of disease and screening of suspected animals. Highest bio-burden of MAP was reported in cattle from UP (Mathura and Agra) and Gujarat in fecal microscopy, where number of cows screened was sufficient. It was also higher in two hilli states (Tamil Nadu and Himanchal Pradesh), though number of cattle screened was limited. Author himself seen large number of clinical cases of cattle in the two states. Since cows cannot be slaughtered in the above states (majority of states except Kerala and North Eastern states) of India, Therefore, the population of low and un-productive cows due to MAP

infection is increasing, since there is no policy on control of JD in cattle and save our precious breeds. Endemicity of MAP is increasing due to repeated passage of bacilli in this species. The findings are in agreement with the productivity, number of clinical cases, ban on cow slaughter etc. In goats, bio-burden was highest in Mathura, moderate in Gujarat and low in Assam. In other states the number of goats screened was lower that too from suspected animals. In sheep, prevalence of MAP was moderate from Tamil Nadu as compared to Rajasthan. Unlike cattle, Unlike cows, buffalo, goats and sheep are undergoing natural selection by sacrifice of low producers. Utility of microscopy is usually under estimated; however in countries with limited resources may be useful screening test at primary or regional diagnostic laboratories. Samples of animals positive in primary laboratories can be sent to specialized laboratories for further confirmation. Our experience with present and previous studies supports that concentration of samples by centrifugation and further decontamination of samples increased the sensitivity of the microscopy. Using ELISA very low prevalence has reported in animals in other parts of the world (Anna Rita *et al*., 2011; Szteyn and Wiszniewska-Laszczych *et al*., 2012). Like fecal microscopy, similar finding were seen in ELISA kit. This study reported 68.4% sero-prevalence of MAP in suspected domestic livestock of different agro-climatic.

In cattle sero-prevalence was higher in UP (Mathura and Agra) than in Gujarat. As compared to earlier studies (29.0%) sero-prevalence of MAP seems to be increasing in large ruminants from Uttar Pradesh using the same kit (Singh *et al*., 2008). Higher sero-prevalence in the present study may be attributed to increase in population of cattle affected with JD. Since cows with low productivity or un-productive cannot be sacrificed for religious reasons. Country has seen sudden spurt in the population of such un-productive cows, which are left on the roads and only some of them may go for illegal slaughter, which is cause of social strife. This is why cows are not longer preferred domestic livestock due to poor salvage value. In goat, sero-prevalence was high in goats from all of the states and as compared to sheep. Goats are more sensitive to MAP infection than sheep. Earlier studies reported pre-dominance of 'Bison type' MAP in domestic and wild ruminants (deer and bison) of North India (Singh *et al*., 2009b; Singh *et al*., 2010a). Present study also showed that domestic livestock from different agro-climatic regions were infected predominantly by 'Bison type' genotype, whereas 'cattle type' biotype was only reported in minority of cases. It may be predicted that 'Bison type' biotype may have high circulation / survival abilities compared to others in Indian conditions that may result in predominance/ higher distribution of this genotype. High bio-burden of may be attributed as one of reason of low animal productivity in India. JD lacks programes on surveillance, monitoring and control of disease. Further, high bio-burden of MAP in present study signifies potential health risk for human beings. We hope finding of present investigation will draw attention of policy makers for developing National programs to fight menace of paratuberculosis in the country.

Conflict of Interest: No conflict of interest to declare.

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