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First mass screening of the human population to estimate the bio-load of *Mycobacterium avium* subspecies *paratuberculosis* in North India

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Bio-load of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was estimated in the first mass screening of human population in Mathura region of South Uttar Pradesh. 48,919 samples were collected between December, 2010 and March, 2013 from Pathology laboratories, 26,390 were screened by indigenous ELISA kit, IS*900* blood and stool PCR, IS*1311* PCR_RE and stool microscopy. From 23,196 serum samples screened by indigenous ELISA, 34.0% were positive for MAP infection (Mathura - 35.4% and Agra 14.2%). Percent prevalence of MAP infection was 28.3, 41.8, 37.4, 29.5, 41.1, 40.7, 42.5, 36.5 and 51.2 in patients suspected for diabetes, liver disorders, anaemia, thyroid, tuberculosis, typhoid, abdominal disorders, inflammatory illness and ion imbalance, respectively. 3093 blood samples screened by IS900 PCR, 8.4% were positive (Mathura - 9.2% and Agra -7.9%). Percent prevalence of MAP was 4.8, 7.0, 20.0, 4.9, 17.8, 7.6 and 12.7 in patients suspected for diabetic, liver disorder, skin disorders, anaemia, Malaria, typhoid and apparently normal individuals, respectively. 101 stool samples screened by microscopy, 5.9% were positive and of these 2.9% were confirmed by IS*900* PCR. IS*1311* PCR_RE bio-typing showed 'Indian Bison Type' was the most prevalent biotype. The study indicated large scale exposure of human population to MAP in the Mathura region of South Uttar Pradesh and 'Indian Bison Type' biotype was most prevalent.

Key words: Blood PCR, bio-load, Crohn's disease, Indian bison type, indigenous ELISA, *Mycobacterium avium* subspecies *paratuberculosis*.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) cause chronic inflammation of intestines in animals and human beings. Chronic inflammatory bowel disease (IBD) or Crohn's disease (CD) share certain clinical and histo-pathological similarities with Johne's disease (JD) and is fast emerging as major disease of public health significance and a potential human infection (Singh et al., 2011a; Singh et al., 2012b). MAP is slow-growing, obligate intracellular fastidious pathogen difficult to grow in culture therefore, it is challenging to detect bacilli in suspected human patients. MAP survives wide range of environmental conditions (pasteurization

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temperature, low pH, and high salt concentration, etc). This superior survival efficiency and dormancy allows pathogen to be more insidious in humans (Whittington et al., 2005). MAP colonizes in animals for years without developing clinical disease. Sub-clinically infected animals shed MAP in their milk; (Shankar et al., 2010) and feces thereby contaminating pastures (Singh et al., 2012c). MAP has been recovered from pasteurized milk (Grant et al., 2002; Millar et al., 1996; Ellingson et al., 2005), infant formula made from pasteurized milk (Hruska et al., 2005), surface water, soil (Hruska et al., 2005; Whan et al., 2005), cow manure ‘‘lagoons’’ that leach into surface water and municipal tap water (Collins et al., 2003), providing multiple routes of transmission to human population. Cow manure in solid and liquid forms is applied as fertilizer in agricultural land (Grewal et al., 2006; Gill et al., 2011).

Studies in India in last two decades showed high prevalence of MAP in domestic (goats, sheep, cattle and buffaloes) and wild (hog deer, blue-bulls, bison, etc) ruminants, other animals (camels, rabbits, etc), primates and human beings (Singh et al., 2012a). Herd prevalence of subclinical MAP in Europe and North America has been reported to range from 21.0 to 70.0%, evidence supporting MAP may be a cause of CD (Rosenfeld and Bressler, 2010). MAP has been detected in the tissues and blood of CD patients with a greater frequency than those without CD (Naser et al., 2004; Sanderson et al., 1992), human breast milk of a patient with CD, positive antibodies to MAP antigens in blood samples of CD patients as compared to controls (Naser et al., 2000). *NOD2/CARD15* gene has previously been shown to be a gene of susceptibility leading to the development of CD (Ogura et al., 2001; Goyette et al., 2007). *NOD2/CARD1* mutations result in a defective innate response to bacterial infection and, possibly, ineffective clearance of intracellular MAP. Earlier studies in India showed sero-prevalence of MAP in suspected human population was estimated as 23.4% from different geographical regions of North India (Singh et al., 2011b). Study reported moderately higher presence of MAP antibodies in human population, which necessitates programs for reducing the bio-load of MAP in the environment and in the animal population (Singh et al., 2011a).

Information on presence and levels of MAP infection in animals and human population outside developed countries is extremely limited (Rajya and Singh, 1961). MAP has also been associated with Type-1 Diabetes (Sechi et al., 2008), autoimmune thyroiditis (D’Amore et al., 2010), multiple sclerosis (Cosu et al., 2013a), autism (Dow, 2011), sarcoidosis (EL-Zaatari et al., 1996), rheumatoid arthritis (Moudgil et al., 1997), autoimmune hepatitis (Miyata et al., 1995), primary biliary cirrhosis (Vilagut et al., 1997), scleroderma (Danieli et al., 1992), Kawasaki disease (Yokota et al., 1993), Behcet’s disease (Direskeneli and Saruhan-Direskeneli, 2003) and Takayasu’s arteritis (Aggarwal et al., 1996). Information on the association of MAP with different human health problems is yet to be recognized and taken seriously by the medical doctors and scientists in India.

Data on genetics and genomics of MAP offered promise that molecular diagnostic strategies may overcome limitations of conventional microbiologic tests used for this fastidious organism (Semret et al., 2005). Insertion element IS*900* is found in 14 to 18 copies per genome of MAP and has been widely used as target sequence for PCR (Moss et al., 1991; Autschbach et al., 2005). The present first large scale screening of human population of Mathura and Agra districts aimed to estimate serological and molecular prevalence of MAP in the human population suspected with different types of health disorders.

In the first large scale screening of human population of the Mathura and Agra regions, the study aimed to determine serological and molecular prevalence of MAP in the human population suspected with different clinical disorders using microscopy, indigenous ELISA test and IS*900* blood and fecal PCR.

MATERIALS AND METHODS

Collection of samples

The work has been approved by the Institute Ethical Committee (IEC) and patients were informed in detail about the sampling and work to be done on their samples (CPCSEA Registration no., 207). A total of 48,919 human samples (Table 1) were collected from 14 different Pathology laboratories located in Mathura and Agra cities from 1st December, 2010 to 31st March, 2013 on daily basis. Of these 48,919 samples, 26,390 (23,196 serum, 3093 blood and 101 stool samples) were randomly screened for the presence of MAP using indigenous ELISA, PCR and microscopy (Table 2).

Indigenous ELISA

Serum samples were screened by ‘Indigenous ELISA kit’ standardized for the screening of human samples using soluble protoplasmic antigen (PPA) prepared from the novel native ‘Indian Bison type’ biotype of MAP strain ‘S 5’ isolated from a terminal case of JD in a Jamunapari goat at CIRG (Sevilla et al., 2005). Serum samples from earlier studies and collected from Crohn’s disease patients confirmed for MAP infection in IS*900* PCR and healthy MAP negative person were used as positive and negative controls, respectively in ELISA. Optical densities (OD) were read at 450 nm. Results were considered accepted if the ratio between mean OD value of the positive and that of negative control was ≥ 4 times. OD values were transformed and expressed as sample to positive (S/P) ratio (Collins, 2002) to determine the status of MAP infection as in the equation below. Serum samples in the S/P ratio range (≥ 0.40) was categories as cut-off and were considered positive for MAP infection.

IS*900* PCR

DNA from human blood samples was isolated and subjected to specific IS*900* PCR. MAP specific primers unique to MAP (IS*900* P90/91) (Millar et al., 1996) were procured. Primers sequences used were:

Table 1. Profile of human clinical samples collected from different pathology laboratories from Mathura and Agra region of south Uttar Pradesh in India between 1 December, 2010 to 31 March, 2013.

Region	Pathology laboratories	Human beings (n)	Samples collected		Paired samples	Stool
			Blood	Serum		
Agra	Arpana pathology	978	893	89	4	4
	Pavan pathology	97	65	65	33	23
	Jivan Jyoti pathology	229	142	149	62	12
	Pankaj pathology	417	109	311	3	3
	Sandhya pathology	979	726	341	88	33
	Dr. Lahiri pathology	80	75	58	53	9
Subtotal A		**2780**	**2010**	**1013**	**243**	**84**
Mathura	New Rangeshwar Pathology Centre	28791	7832	22522	1563	10
	Brij centre pathology	989	424	709	144	2
	Rama path.	804	335	592	123	2
	Sushila Hospital	61	36	36	11	-
	Varsha pathology	408	162	275	29	-
	Pathak pathology	19	19	5	5	-
	Swarna Jayanti Hospital	1053	463	749	159	3
	Mathura laboratory	7795	4901	5009	2115	-
Subtotal B		**39920**	**14172**	**29897**	**4149**	**17**
Total		**42400**	**15882**	**32936**	**4392**	**101**

Table 2. Region-wise distribution of blood and serum samples processed from different pathology laboratories from Agra and Mathura region.

Places	Samples (n)			Total
	Serum	Blood	Stool	
Mathura	21,649	1,130	17	22796
Agra	1,547	1,963	84	3594
Sub-total	23,196	3093	101	26390
Total	46,392	6186	202	-

$$\text{S/P ratio} = \frac{\text{O.D 450 nm of the sample} - \text{O.D 450 nm of the negative control}}{\text{O.D 450 nm of the positive control} - \text{O.D 450 nm of the negative control}}$$

1. Forward primer- P90 5'- GAA GGG TGT TCG GGGCCGTCGCTTAGG -3'
2. Reverse primer- P91 5'- GGC GTT GAG GTC GATCGC CCA CGT GAC -3'

Briefly, PCR was set up in volume of 50 µl, using 1 to 5 ng template DNA, 5 µl of 10×PCR buffer, 2.5 mM $MgCl_2$, 0.2 mMdNTPs, 10 pmol of each primer and 5 U *Taq* polymerase. Thermal cycling conditions were set as initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 63°C for 10s, extension at 72°C for 10 s and final extension at 72°C for 3 min. Product size of 413 bp was considered positive, after separation on 2.0% agarose gel stained with ethidium bromide.

IS*1311* PCR

IS*900* PCR positives were subjected to IS*1311* PCR using M*56* and M119 primers (Sevilla et al., 2005) with some modifications. Briefly, PCR was set up in volume of 25 µl, using 0.5 to 1.0 ng template DNA, 2.5 ml of 10×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U Taq (Promega, Madison, WI). Thermal cycling was as follows: initial denaturation at 94°C for 3 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. An amplicon size of 608 bp was considered positive in IS*1311* PCR, after separation on a 2.0% agarose gel stained with ethidium bromide.

IS*1311* PCR-restriction endonuclease analysis (REA)

IS*1311* PCR-REA was carried out according to Sevilla et al., (2005). Briefly, the reaction was carried out in a volume of 30 µl, containing 20 µl positive IS*1311* PCR product, 3 µl 10×buffer and 2 U of each endonuclease *HinfI* and *MseI* (Fermentas, USA). The reaction mixture was incubated at 37°C for 1.5 h. Band patterns were visualized after electrophoresis on 4.0% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted according to Whittington et al., (2001).

Microscopic Examination and isolation of Bacterial DNA from stool samples

Two grams of stool sample was grounded in sterilized distilled water (10 to 12 ml) in sterilized pestle and mortar. Grounded

Table 3. Status of MAP infection in the human population of Mathura district using Indigenous ELISA test based on S/P ratio method of Collins (2002).

Samples screened *(n)*	S/P Ratio	Status of MAP infection	Sero-status *n* (%)	Cummulative total
23,196	0.00-0.09	Negative	6147 (26.5)	Total Negative 15303 (66.0%)
	0.10-0.24	Suspected	4582 (19.7)	
	0.25-0.39	Low positive	4574 (19.7)	
	0.40-0.99	Positive	6482 (27.9)	Total Positive 7893 (34.0%)
	1.0-10.0	Strong Positive	1401 (6.0)	

Table 4A. Sero-status of MAP infection in the human population of Mathura district suspected with non-infectious clinical conditions.

S/N	Clinical profile of samples	Samples *(n)*	Strong positives *n* (%)	Positives *n* (%)	Total positives *n* (%)
1	Diabetes	9816	420 (4.2)	2360 (24.0)	2780 (28.3)
2	Liver disorder	2219	147 (6.6)	781 (35.1)	928 (41.8)
3	Anemia	2416	209 (8.6)	696 (28.8)	905 (37.4)
4	Thyroid Disorder	3024	149 (4.9)	746 (24.6)	895 (29.5)
5	Ion Imbalance	995	133 (13.3)	377 (37.8)	510 (51.2)
6	Abdominal Disorder	54	03 (5.5)	20 (37.0)	23 (42.5)
7	Lipid Profile	265	03 (1.1)	121 (45.6)	124 (46.8)
8	Others (Urea,UA,LH,PRL)	467	24 (5.1)	115 (24.6)	139 (29.7)
	Sub Total	19,256	1088 (5.6)	5216 (27.0)	6304 (32.7)

Table 4B. Sero-status of MAP infection in the human population of Mathura district suspected with infectious clinical conditions.

S/N	Clinical Profile of samples	Samples *n*	Strong Positives *n* (%)	Positives *n* (%)	Total Positives *n* (%)
1	Typhoid	2824	200 (7.0)	950 (33.6)	1150 (40.7)
2	Tuberculosis	316	35 (11.0)	95 (30.0)	130 (41.1)
3	Inflammatory illness	230	25 (10.8)	59 (25.6)	84 (36.5)
4	Others (VDRL,TORCH)	570	53 (9.2)	172 (30.1)	225 (39.4)
	Sub Total	3940	313 (7.9)	1266 (32.1)	1589 (40.3)

material was centrifuged at 1557 × g for 1 h at room temperature; smears prepared from middle layer, stained with Ziehl-Neelsen (ZN)staining and were observed under oil immersion for presence of pink staining acid-fast short rods indistinguishable to MAP. Middle layer was also decontaminated using 0.9% hexa decyl pyridinium chloride (HPC) (Singh et al., 1996). After decontamination, DNA was isolated from the sediment pellet according to method of Whipple et al., (1991) with some modifications. DNA from positive samples by microscopy, were also screened using IS*900* PCR .

RESULTS

Indigenous ELISA kit

23,196 serum samples were screened, 7893 (34%) were positive (Table 3) for the presence of anti-MAP antibodies/MAP infection. From Mathura (*n*=21,649) and Agra (*n*=1,547) districts, 35.4 and 14.2% were positive, respectively. Of the various suspected non-infectious and infectious pathological conditions for which the serum samples were submitted, 28.3, 41.8, 37.4, 29.5, 41.1, 40.7, 42.5, 36.5 and 51.2% were positive for MAP infection from the cases of diabetes, liver disorder, anemia, thyroid disorders, tuberculosis, typhoid, abdominal disorders, inflammatory illness, ion imbalance, respectively (Table 4A and 4B). Age-wise persons in the age group of >40 yr showed higher prevalence of MAP, however sex-wise, there was no significant difference between male and female population with respect to MAP infection (Table 5).

IS*900* Blood PCR

Screening of 3093 blood samples by IS*900* blood PCR, 262 (8.4%) were positive for MAP infection. From Mathura (*n*=1130) and Agra (*n*=1963) regions, 9.2 and 7.9% blood samples were positive, respectively. Of the various suspected infectious and non-infectious pathological conditions for which the blood samples were submitted to the pathologies laboratories, 4.8, 7.0, 20.0,

Table 5. Sex-wise sero-status of MAP infection in the human population of Mathura and Agra regions.

Regions	Males		Females		Total *n* (%)
	Samples	Positives *n* (%)	Sample *(n)*	Positive *n* (%)	
Mathura	11289	4054 (35.9)	10,360	3618 (34.9)	7672/21649 (35.4%)
Agra	757	102 (13.4)	790	118 (14.9)	220/1547 (14.2%)
Subtotal	12046	4158 (34.5%)	11150	3736 (33.5%)	7894/23,196 (34.0%)

Table 6. Detection of MAP infection by IS*900* blood PCR in the human population of Mathura district suspected for non-infectious and infectious clinical conditions

S/N	Sampling parameter	Samples processed (*n*)	Positives *n* (%)
Non-infectious health problems			
1	Lipid Profile	121	5 (4.1)
2	Diabetes	451	22 (4.8)
3	Liver disorder	71	5 (7.0)
4	Kidney Dysfunction	70	0 (0)
5	Thyroid Disorder	63	0 (0)
6	Anemia	749	37 (4.9)
	Sub-Total	1525	69 (4.5)
Infectious diseases			
7	Typhoid	39	3 (7.6)
8	Tuberculosis	10	0 (0)
9	Others (VDRL, TORCH)	16	0 (0)
10	Skin disorder	5	1 (20.0)
11	Malaria	56	10 (17.8)
	Sub-Total	126	14 (11.1)
Others			
12	Normal Healthy Individuals	1246	159 (12.7)
13	Blood grouping	196	20 (10.2)
	Sub Total	1442	179 (12.4)
Total		**3093**	**262 (8.4)**

4.9, 17.8, and 7.6% positive blood samples belonged to cases of diabetes, liver disorders, skin disorders, anemia, malaria and typhoid, respectively (Table 6). Screening of blood samples of 1442 apparently normal individuals, 12.4% were positive in IS*900* blood PCR (Table 6). Sex-wise there was no significant difference between male and female population with respect to MAP infection (Table 7).

Microscopic examination and isolation of DNA for IS*900* PCR on stool samples

Of the 101 blood samples screened, 5.9% (6) and 2.9% (3) were positive in microscopy and IS*900* PCR, respectively. Of 17 stool samples from Mathura region none was positive by microscopy. However, of the 84 stool samples from Agra region, 7.1% (6/84) were positive for acid-fast bacilli (AFB) indistinguishable to MAP (Table 8). Of these AFB positive samples; 3.5% (3/84) were positive by IS*900* PCR (Table 7, Figure 1). Geno-typing of representative IS*900* PCR positive DNA showed presence of 'Indian Bison Type' biotype in the two regions.

DISCUSSION

Present study was first attempt to estimate bio-load of MAP infection in the human population of Mathura and Agra regions by large scale screening of human samples submitted to different pathological laboratories in the

Table 7. Sex-wise presence of MAP infection by 'IS*900* PCR in blood sample'.

Region	Males		Females		Total *n* (%)
	Samples *(n)*	Positives (%)	Samples *n)*	Positives *n* (%)	
Mathura	503	58 (11.5)	627	47 (7.4)	105/1130 (9.2)
Agra	936	85 (9.0)	1027	72(7.0)	157/1963 (7.9)
Subtotal	1439	143 (9.9)	1654	119 (7.1)	262/3093 (8.4)

Table 8. Screening of stool sample by microscopy and IS*900* PCR.

Region	Stool samples *(n)*	Positive *n* (%)	
		Microscopy	IS*900* PCR
Agra	84	6 (7.1)	3 (3.5)
Mathura	17	0 (0)	0 (0)
Total	101	6 (5.9%)	3 (2.9%)

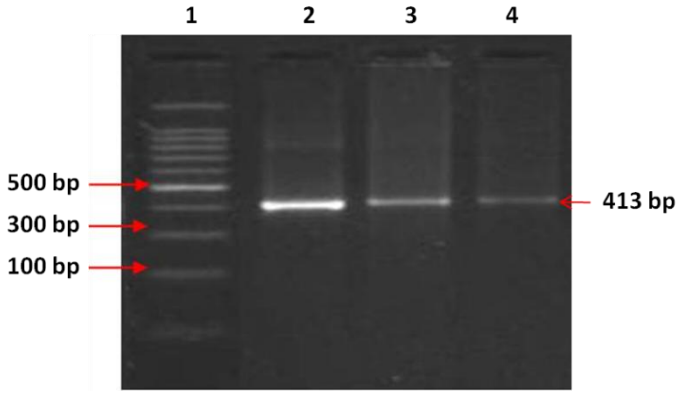

Figure 1. MAP specific amplicons (413bp) using IS*900* specific primers. Lane 1: 100bp ladder, lane 2: Positive control, lane 3-4: DNA samples.

region. Our earlier pilot studies has confirmed the presence of MAP infection in the confirmed and suspected cases of Inflammatory Bowel Diseases (Crohn's disease), animal workers suffering with chronic colitis (suspected for IBD), animal and non-animal keepers living on the periphery of big cities and apparently normal human beings (Singh et al., 2011a; Shisodiya et al., 2009). Present Indian medical science is more inclined towards clinical management of IBD & CD and totally disregards the role of MAP in these clinical conditions. In general, little attention is paid towards research especially against infections of animal origin, e.g., MAP. In absence of interest in MAP it was difficult to get samples (biopsies, blood, serum etc.) from cases of IBD/CD. Therefore in the present study, the strategy adopted was to estimate presence and level of MAP infection by first large scale screening of human population, irrespective of any particular disease condition from Mathura and Agra region. For this purpose serum and blood samples were collected from different pathology laboratories, where blood, serum and stool samples were submitted for various infectious and non-infectious health disorders. Previously developed 'indigenous ELISA test' for other livestock species (Collin et al., 2005, Pruvot et al., 2013) has been standardized for the screening of human serum samples (Singh et al., 2011c). Using 'indigenous ELISA kit' in the present investigation indicated high (34%) bio-load of MAP in the human population indicating heavy exposure to MAP infection. Studies by other workers also reported presence of MAP in human population particularly in patients of CD and diabetes (Greenstein et al., 2003; Bitti et al., 2012). A population based study reported 35% sero-positivity rates however; there was no difference in rates between CD patient, UC patients and healthy controls (Collins et al., 2000). Similarly in present findings also there was no significant difference in the rates of MAP infection between patients suspected for suffering with infectious (32.7%) and non-infectious (40.3%) clinical conditions. Earlier studies by Singh et al., (2008) reported high sero-positivity in CD patients (100%), animal attendants (75%) and apparently normal human beings (38%). In a sero-survey of animal keepers suspected and not-suspected for CD showed that 12.9 and 4.2% were positive by the indigenous ELISA test (Shisodiya et al., 2009; Singh et al., 2011a) while screening of animal attendants who worked with goatherds endemic for MAP infection had higher prevalence of MAP infection as compared with person with no history of contact with animals. In another study by Singh et al. (2011c), Sero-prevalence of MAP was higher in CD patients (80%), as compared to ulcerative colitis patients (4.5%) and apparently normal persons (15.3%) using 'indigenous adsorbed ELISA test. The further reported sero-prevalence of MAP in another group of apparently normal human beings as 23.4%. Geographical region-wise, 34.0, 33.3, 32.8, 25.0, 23.0, 17.7% and 12.5% serum

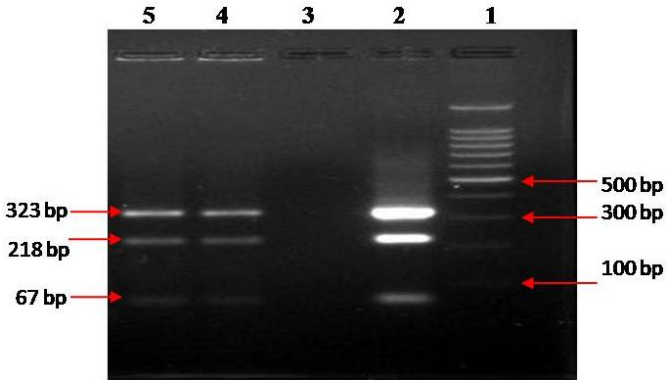

Figure 2. IS*1311* PCR-REA analysis. Lane 1: 100bp DNA ladder, lane 2: Positive control DNA, lane 3: Negative control, lane 4 and 5: Digested DNA sample ('Indian Bison Type')

samples were positive from the states of Punjab, Uttarakhand, New Delhi, Himanchal Pradesh, Haryana, Uttar Pradesh and Jammu and Kashmir, respectively. Presence of higher bio-load of MAP in animals (domestic and wild ruminants) (Kumar et al., 2010) and animal products (milk and milk products) (Shankar et al., 2010) indicated animals are source of MAP infection to human population directly (by contact and consumption of animal products) and indirectly (through contact). Animal keepers and attendants stand at higher risk and falling easy prey to exposure of MAP through heavy to very heavy load of MAP bacilli in animals and environment.

The study also analysed 3091 blood samples of human beings from Mathura region using IS*900* blood PCR, wherein 8.4% (262) were found positive for MAP infection. Of these 262 positive human beings, 4.5, 11.1 and 12.4%% positive samples were from non-infectious, infectious clinical conditions and apparently normal individuals, respectively. Skin disorders, malaria, typhoid, liver disorder, anaemia, diabetes and lipid profiles were the major suspected clinical conditions for which the blood samples were submitted to pathology laboratories and found positive for MAP infection in IS*900* blood PCR. Whereas in ELISA, serum samples were positive for all the suspected clinical conditions for which the samples were submitted (Table 4a and Table 4b).

Screening of stool samples by microscopy and IS*900* PCR, 5.9 and 2.9% were positive for MAP respectively (Table 8). Presence of acid fast bacilli indistinguishable to MAP in stool samples using microscopy was an interesting findings, which we have also reported in our previous studies (Singh et al., 2008). However, studies in other parts of the world reported cell wall deficient (CWD) forms of MAP in human beings (Greenstein, 2003). Presence of heavy load of MAP in human stools should be considered alarming. Recently, it has been reported that MAP antigens have the capacity to induce colitis in mice (Momotani et al., 2012). Further investigations are required if these AFB have any role in developing the disease.

Naser et al. (2004) also detected MAP bacilli in blood of 50% patient with CD and 22% of patients with UC. Presence of MAP in blood, suggests that it may be distributed to different organs and may play pathological role in at different sites of infection. Presence of MAP in blood has been directly related with etiological role in CD (Naser et al., 2009). However, presence of MAP in blood of healthy individuals in present study has been reported earlier also (Singh et al., 2011c) has given rise to controversies and food for especially those people who contradicts the role of MAP in CD.

However, recent advancements in MAP research indicated the presence and role of MAP in patients with various diseases such as Type-1 Diabetes (Sechi et al., 2008), autoimmune thyroiditis (D'Amore et al., 2010), multiple sclerosis (Cossu et al., 2013a), autism (Dow, 2011), sarcoidosis (EL-Zaatari et al., 1996) and autoimmune arthritis (Moudgil et al., 1997). Our study correlates with the finding of some authors that have claimed the association of MAP infection with autoimmune disorder viz. Type-1 Diabetes and thyroid disorder etc.

Earlier, it has been reported that mycobacterial heat shock protein (HSP65), shares sequential and conformational elements with several human proteins and it can be predicted that by molecular mimicry mecha-nisms, MAP can stimulate auto-antibodies resulting auto-immune disorders like CD, type 1 diabetes, autoimmune (Hashimoto's) thyroiditis, and multiple sclerosis etc. Mycobacterial HSP65 has also been implicated in the pathogenesis of rheumatoid arthritis, autoimmune hepatitis, primary biliary cirrhosis, scleroderma and Kawasaki disease (Dow, 2012). It has also been pre-dicted that MAP HSP60/65 triggers anti-GAD (pancreatic glutamic acid decarboxylase) antibodies that destroy the pancreas (Jones et al., 1993; Naser et al., 2013). Further it has been reported that sera from children with type I diabetes shows strong sero-reactivity to MAP specific protein MAP3738c (Cossu et al., 2013b). Further, it has been shown that sera from diabetes patients react against MAP Hsp65 protein (Naser et al., 2013). In our present study also, 28.3 (2780/9816) and 4.8% (22/451) patients suspected for diabetes were found positive for MAP infection in 'indigenous ELISA and IS*900* blood PCR, respectively.

IS*1311* PCR_RE method specifically designed for the bio-typing of MAP by Whittington et al. (2001) was used to characterize native strains and in India, a new biotype of MAP 'Indian Bison type' has been reported as principal biotype infecting different animal species, animal products and human beings (Shankar et al., 2010; Singh et al., 2013). This assay (Figure 2) helped us to further give an insight that this particular biotype of MAP has accumulated genetic differences compared to MAP 'K 10' and other international MAP isolates (Fiocchi, 1998).

Unlike JD in animals, MAP has been found as cell wall deficient form in CD patients (Greenstein, 2003); however, in India, cell wall containing MAP has been recovered from animal healthcare workers (suspected for CD). In later studies, these cell walls containing MAP were genotyped as 'Indian bison type' genotype of MAP (Singh et al., 2009a). These findings indicated that 'Indian bison type' strain may be capable of initiating disease that manifests similarly to JD in animals. 'Indigenous ELISA' using PPA from 'Indian bison type' biotype MAP of goat origin was superior when compared with imported commercial ELISA kits for screening of animals was also useful in screening of human samples for MAP infection (Singh et al., 2011c). Presence of MAP in patients suspected for infectious and non-infectious clinical conditions showed that besides association with Inflammatory bowel disease (Crohn's disease), MAP may also play an important role in other health disorders and cases of colitis and other abdominal ailment in India. Due to the presence of MAP in the milk of animals (Shankar et al., 2010) and human (Naser et al., 2000) it is highly likely that MAP will be passed on to the next generation through milk, thereby creating endemicity of infection in a population or colony. In India people may get infected by other means as MAP has also been reported from environment soil and river water (Singh et al., 2012c) and abdominal disorders very common in India. Presence of MAP in human population with various suspected pathological conditions and from apparently normal individuals indicated large scale exposure of human population to MAP in Mathura region of South Uttar Pradesh in India. Both serological and molecular tests helped establishing presence of MAP organism in clinical samples and possible association with various pathological and physiological conditions.

Conclusion

The superior survivability of MAP allows the pathogen to be more insidious for human health. Despite the full genome sequencing, little information is available about the prevalence of this pathogen under the Indian condition. The present work has been carried out by combining both serological and molecular tools viz., faecal culturing, Indigenous ELISA kit, blood PCR targeting IS*900* and IS*1311* sequences as well as PCR_RE, in order to detect in better way the organism in clinical samples of human patients and find out its association with various pathological and physiological conditions, so that the researchers and diagnosticians can have a better understanding of the epidemiological status of the disease, CD. Present study reports high burden of MAP in human population and further studies are required to address the heavy load of MAP in different disease conditions in North Indian population.

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