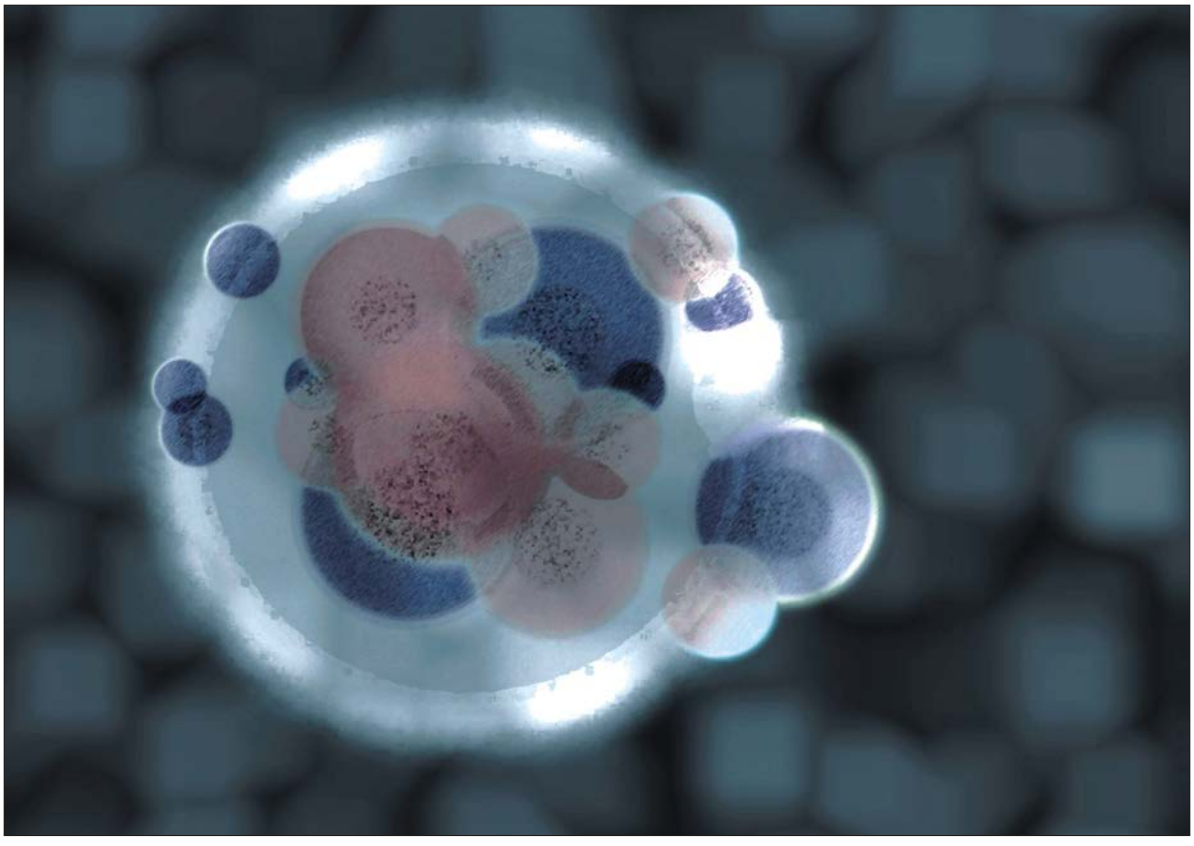

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First Mass Screening of the Human Population to Estimate the Bio-load of *Mycobacterium avium* Subspecies *paratuberculosis* in North India

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Abstract: Bio-load of *Mycobacterium avium* subspecies *paratuberculosis* was estimated in the first mass screening of human population in Mathura region of South Uttar Pradesh. Of the 48,919 samples 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_REA and stool microscopy. Of the 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 disorder, tuberculosis, typhoid, abdominal disorders, inflammatory illness and ion imbalance, respectively. Of 3093 blood samples screened by IS*900* 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. Of the 101 stool samples screened by microscopy, 5.9% were positive and of these 2.9% were confirmed by IS*900* PCR. IS*1311* PCR_REA bio-typing showed 'Indian Bison Type' was the most prevalent biotype. Study indicated large scale exposure of human population to MAP infection in the Mathura region of South Uttar Pradesh and like in animals'Indian Bison Type' was the most prevalent biotype of MAP infecting human beings in this region.

Key words: 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 MAP is fast emerging as major pathogen of public health significance and a potential human infection (Singh *et al*., 2008, 2011a, 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 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 may colonize in animals for years without developing

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clinical disease. Sub-clinically infected animals shed MAP in their milk (Singh *et al*., 2009a; Shankar *et al*., 2010) and feces thereby contaminating new born calves and 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, 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 last two decades in India 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*., 2010, 2011b, 2012a). Herd prevalence of subclinical MAP in Europe and North America has been reported to range from 21.0-70.0%, an 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*., 2011a). 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*., 2008, 2011a).

Information on presence and levels of MAP infection in animals and human population outside developed countries is extremely limited (Rajya *et al*., 1961). MAP has also been associated with 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), 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).

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 of suffering with different clinical disorders using microscopy, indigenous ELISA test and IS*900* blood and fecal PCR test.

MATERIALS AND METHODS

Ethical approval: The work has been approved by the Institute Animal Ethical Committee (IAEC) and patients were informed in detail about the sampling and work to be done on their samples (Committee for the purpose of control and supervision of experiments on Animals Registration No. 207).

Collection of samples: A total of 48,919 human samples (Table 1) were collected from 14 different Pathology laboratories located in Mathura and Agra cities from 1st Dec 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 as per Sevilla *et al*. (2005) and Singh *et al*. (2009a). 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

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 Dec 2010 and 31 March 2013

			Samples collected			
Region	Pathology laboratories	Human beings (n)	Blood	Serum	Paired samples	Stool
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

	Samples (n)			
Places	Serum	Blood	Stool	Total
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	

controls, respectively. 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 as per Collins (2002) to determine the status of MAP infection as per following equation. Serum samples in the S/P ratio range (≥0.40) was categorised as cut-off and were considered positive for MAP infection:

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

IS*900* PCR: DNA from human blood samples was isolated and subjected to specific IS*900* PCR as per Singh *et al.* (2010). MAP specific primers unique to MAP (IS*900* P90/91) as per Millar *et al.* (1996) were procured. Primers sequences used were forward primer-P90 5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3' reverse primer-P91 5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3.

Briefly, PCR was set up in volume of 50 μL, using 1.0-5.0 ng template DNA, 5 μL of 10X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer and 5U Taq polymerase. Thermal cycling conditions were set as initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 7 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 M56 and M119 primers as per Sevilla *et al.* (2005) with some modifications. Briefly, PCR was set up in volume of 25 μL, using 0.5-1.0 ng template DNA, 2.5 mL of 10X 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 sec, 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 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, 3 μL 10X 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 as per 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 (10-12 mL) in sterilized pestle and mortar. Grounded material was centrifuged at 1557 x g for 1 h at room temperature; smears

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%)
	1.0-10.00	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

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

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

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

	Males		Females		
Regions	Samples	Positives n (%)	Sample (n)	Positive n (%)	Total 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)

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) as per Singh *et al.* (1996). After decontamination DNA was isolated from the sediment pelleted as per method of Whipple *et al.* (1991) with some modifications (Singh *et al.*, 1998). DNA from positive samples by microscopy, were also screened using IS*900* PCR as per Singh *et al.* (2010).

RESULTS

Indigenous ELISA kit: Of 23,196 serum samples screened, 7893 (34.0%) were positive for the presence of anti-MAP antibodies/MAP infection (Table 3). 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 b). Age-wise persons in the age group of >40 year 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, 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).

Table 6: Detection of MAP infection by blood IS*900* 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 (0.0)
5	Thyroid disorder	63	0 (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 (0.0)
9	Others (VDRL, TORCH)	16	0(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)

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

	Males		Females		
Region	Samples (n)	Positives (%)	Samples (n)	Positives n (%)	Total 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

		Positives n (%)	
Region	Stool samples (n)	Microscopy	IS*900* PCR
Agra	84	6 (7.1)	3 (3.5)
Mathura	17	0 (0)	0 (0.0)
Total	101	6 (5.9%)	3 (2.9%)

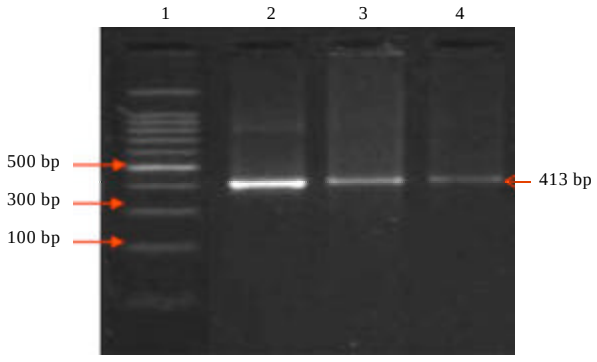

Fig. 1: MAP specific amplicons (413 bp) using IS*900* specific primers. Lane 1: 100 bp ladder, Lane 2: Positive control, Lane 3-4: DNA samples

Microscopic examination and isolation of DNA for IS*900* PCR on stool samples: Of the 101 blood samples screened, 5.9 (n = 6) and 2.9% (n = 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. Of these AFB positive samples; 3.5% (3/84) were positive by IS*900* PCR (Table 8, Fig. 1). Geno-typing of representative IS*900* PCR positive DNA showed presence of 'Indian Bison Type' biotype in the two regions (Fig. 2).

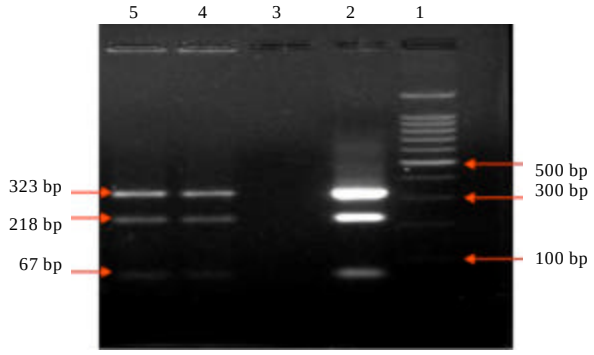

Fig. 2: IS *1311* PCR-REA analysis, Lane 1: 100 bp DNA ladder, Lane 2: Positive control DNA, Lane 3: Negative control and Lane 4 and 5: Digested DNA sample ('Indian Bison type')

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 two regions. 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 (Sevilla *et al*., 2005; Singh *et al*., 2011a, 2012b; Sishodia *et al*., 2009). Present Indian medical science is more inclined towards clinical management of IBD and 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 regions. 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 (Collins *et al*., 2005; Singh *et al*., 2009b; Pruvot *et al*., 2013) has been standardized for the screening of human serum samples (Singh *et al*., 2007, 2011c). Using 'indigenous ELISA kit' in the present investigation reported high (34.0%) 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, 2003; Bitti *et al*., 2012). A population based study reported 35.0 % 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 Sevilla *et al*. (2005) reported high sero-positivity in CD patients (100.0%), animal attendants (75.0%) and apparently normal human beings (38.0%). 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 (Sishodia *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 persons with no history of contact with animals. In another study by Singh *et al*. (2011c), sero-prevalence of MAP was higher in CD patients (80.0%), as compared to ulcerative colitis patients (4.5%) and apparently normal persons (15.3%) using 'indigenous adsorbed ELISA test. They 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 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 (Singh *et al*., 2010; 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% (n = 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 b).

Screening of stool samples by microscopy and IS*900* PCR, 5.9 and 2.9% were positive for MAP, respectively. 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 (Sevilla *et al*., 2005). 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 50.0% patients 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 mechanisms, MAP can stimulate auto-antibodies resulting autoimmune 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 predicted 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.*, 2009b, 2013). This assay 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.*, 2009b). 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 (Singh *et al.*, 2007) 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 whole genome sequencing, little information is available about the prevalence of this pathogen under the Indian conditions. 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_REA, 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, like CD. Present study reports high bio-load of MAP in human population of Mathura district and further studies are required to address the heavy load of MAP in different disease conditions in the human population of North India.

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REFERENCES

Aggarwal, A., M. Chag, N. Sinha and S. Naik, 1996. Takayasu's arteritis: Role of *Mycobacterium tuberculosis* and its 65 kDa heat shock protein. Int. J. Cardiol., 55: 49-55.

Autschbach, F., S. Eisold, U. Hinz, S. Zinser and M. Linnebacher *et al*., 2005. High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* IS*900* DNA in gut tissues from individuals with Crohn`s disease. Gut, 54: 944-949.

Bitti, M.L.M., S. Masala, F. Capasso, N. Rapini, S. Piccinini and F. Angelini *et al*., 2012. *Mycobacterium avium* subsp. *paratuberculosis* in an Italian cohort of type 1 diabetes pediatric patients. Clin. Dev. Immunol., Vol. 2012. 10.1155/2012/785262

Collins, M.T., 2002. Interpretation of a commercial bovine *paratuberculosis* enzyme-linked immunosorbent assay by using Likelihood Ratios. Clin. Vaccine Immunol., 9: 1367-1371.

Collins, M.T., 2003. *Paratuberculosis*: Review of present knowledge. Acta. Vet. Scand., 44: 217-221.

Collins, M.T., G. Lisby, C. Moser, D. Chicks and S. Christensen *et al*., 2000. Results of multiple diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. J. Clin. Microbiol., 38: 4373-4381.

Collins, M.T., S.J. Wells, K.R. Petrini, J.E. Collins, R.D. Schultz and R.H. Whitlock, 2005. Evaluation of five antibody detection tests for diagnosis of bovine *paratuberculosis*. Clin. Vaccine Immunol., 12: 685-692.

Cossu, A., E. Ferrannini, P. Fallahi, A. Antonelli and L.A. Sechi, 2013a. Antibodies recognizing specific *Mycobacterium avium* subsp. *paratuberculosis*'s MAP3738c protein in type 1 diabetes mellitus children are associated with serum Th1 (CXCL10) chemokine. Cytokine, 61: 337-339.

Cossu, D., S. Masala and L.A. Sechi, 2013b. A Sardinian map for multiple sclerosis. Future Microbiol., 8: 223-232.

D'Amore, M., S. Lisi, M. Sisto, L. Cucci and C.T. Dow, 2010. Molecular identification of *Mycobacterium avium* subspecies *paratuberculosis* in an Italian patient with Hashimoto's thyroiditis and Melkersson-Rosenthal syndrome. J. Med. Microbiol., 59: 137-139.

Danieli, M.G., M. Candela, A.M. Ricciatti, R. Reginelli, G. Danieli, I.R. Cohen and A. Gabrielli, 1992. Antibodies to mycobacterial 65 kDa heat shock protein in systemic sclerosis (scleroderma). J. Autoimmunity, 5: 443-452.

Direskeneli, H. and G. Saruhan-Direskeneli, 2003. The role of heat shock proteins in Behcet's disease. Clin. Exp. Rheumatol., 21: S44-S48.

Dow, C.T., 2011. *Mycobacterium paratuberculosis* and autism: Is this a trigger? Med. Hypotheses, 77: 977-981.

Dow, C.T., 2012. *M. paratuberculosis* heat shock protein 65 and human diseases: Bridging infection and autoimmunity. Autoimmune Dis., Doi: 0.1155/2012/150824

El-Zaatari, F.A., S.A. Naser, D.C. Markesich, D.C. Kalter, L. Engstand and D.Y. Graham, 1996. Identification of *Mycobacterium avium* complex in sarcoidosis. J. Clin. Microbiol., 34: 2240-2245.

Ellingson, J.L.E., J.L. Anderson, J.J. Koziczkowski, R.P. Radcliff, S.J. Sloan, S.E. Allen and N.M. Sullivan, 2005. Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. J. Food Protect., 68: 966-972.

Fiocchi, C., 1998. Inflammatory bowel disease: Etiology and pathogenesis. Gastroenterology, 115: 182-205.

Gill, C.O., L. Saucier and W.J. Meadus, 2011. *Mycobacterium avium* subsp. *paratuberculosis* in dairy products, meat and drinking water. J. Food Protect., 74: 480-499.

Goyette, P., C. Labbe, T.T. Trinh, R.J. Xavier and J.D. Rioux, 2007. Molecular pathogenesis of inflammatory bowel disease: Genotypes, phenotypes and personalized medicine. Ann. Med., 39: 177-199.

Grant, I.R., E.I. Hitchings, A. McCartney, F. Ferguson and M.T. Rowe, 2002. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows milk. Applied Environ. Microbiol., 68: 602-607.

Greenstein, R.J., 2003. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis and Johne's disease. Lancet Infect. Dis., 3: 507-514.

Grewal, S.K., S. Rajeev, S. Sreevatsan and F.C. Michel, 2006. Persistence of *Mycobacterium avium* subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing and liquid storage of dairy manure. Applied Environ. Microbiol., 72: 565-574.

Hruska, K., M. Bartos, P. Kralik and I. Pavlik, 2005. *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant milk: *Paratuberculosis* in cattle-the public health problem to be solved. Vet. Med., 50: 327-335.

Jones, D.B., A.F.W. Coulson and G.W. Duff, 1993. Sequence homologies between hsp60 and autoantigens. Immunol. Today, 14: 115-118.

Kumar, S., S.V. Singh, A.V. Singh, P.K. Singh, J.S. Sohal and A. Maitra, 2010. Wildlife (*Boselaphus tragocamelus*)-small ruminant (goat and sheep) interface in the transmission of Bison type genotype of *Mycobacterium avium* subspecies *Paratuberculosis* in India. Comp. Immunol. Microbiol. Infect. Dis., 33: 145-159.

Millar, D., J. Ford, J. Sanderson, S. Withey, M. Tizard, T. Doran and J. Hermon-Taylor, 1996. IS*900* PCR to detect *Mycobacterium paratuberculosis* in retail supplies of whole pasteurized cows milk in England and Wales. Applied Environ. Microbiol., 62: 3446-3452.

Miyata, M., A. Kogure, H. Sato, E. Kodama, H. Watanabe *et al*., 1995. Detection of antibodies to 65 KD heat shock protein and to human superoxide dismutase in autoimmune hepatitis-molecular mimicry between 65 KD heat shock protein and superoxide dismutase. Clin. Rheumatol., 14: 673-677.

Momotani, E., N.M. Romona, K. Yoshihara, Y. Momotani, M. Hori and H. Ozaki, 2012. Molecular pathogenesis of bovine *paratuberculosis* and human inflammatory bowel diseases. Vet. Immunol. Immunopathol., 148: 55-68.

Moss, M.T., E.P. Green, M.L. Tizard, Z.P. Malik and J. Hermon-Taylor, 1991. Specific detection of *Mycobacterium paratuberculosis* by DNA hybridisation with a fragment of the insertion element IS*900*. Gut, 32: 395-398.

Moudgil, K.D., T.T. Chang, H. Eradat, A.M. Chen, R.S. Gupta, E. Brahn and E.E. Sercarz, 1997. Diversification of T cell responses to carboxy-terminal determinants within the 65-kD Heat-shock protein is involved in regulation of autoimmune arthritis. J. Exp. Med., 185: 1307-1316.

Naser, S.A., D. Schwartz and I. Shafran, 2000. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. Am. J. Gastroenterol., 95: 1094-1095.

Naser, S.A., G. Ghobrial, C. Romero and J.F. Valentine, 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet, 364: 1039-1044.

Naser, S.A., M.T. Collins, J.T. Crawford and J.F. Valentine, 2009. Culture of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from the blood of patients with Crohn's disease: A follow-up blind multi center investigation. Open Inflam. J., 2: 22-23.

Naser, S.A., S. Thanigachalam, C.T. Dow and M.T. Collins, 2013. Exploring the role of *Mycobacterium avium* subspecies *paratuberculosis* in the pathogenesis of type 1 diabetes mellitus: A pilot study. Gut. Pathog., 5:14.

Ogura, Y., D.K. Bonen, N. Inohara, D.L. Nicolae and F.F. Chen *et al*., 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature, 411: 603-606.

Pruvot, M., T.L. Forde, J. Steele, S.J. Kutz, J. de Buck, F. van der Meer and K. Orsel, 2013. The modification and evaluation of an ELISA test for the surveillance of *Mycobacterium avium* subsp. *paratuberculosis* infection in wild ruminants. BMC. Vet. Res.

Rajya, B.S. and C.M. Singh, 1961. Studies on the pathology of Johne's disease in sheep. III. Pathologic changes in sheep with naturally occurring infections. Am. J. Vet. Res., 22: 189-203.

Rosenfeld, G. and B. Bressler, 2010. *Mycobacterium avium paratuberculosis* and the etiology of Crohn's disease: A review of the controversy from the clinician's perspective. Can. J. Gastroenterol., 24: 619-624.

Sanderson, J.D., M.T. Moss, M.L. Tizard and J. Hermon-Taylor, 1992. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Gut, 33: 890-896.

Sechi, L.A., V. Rosu, A. Pacifico, G. Fadda, N. Ahmed and S. Zanetti, 2008. Humoral immune responses of type 1 diabetes patients to *Mycobacterium avium* subsp. *paratuberculosis* lend support to the infectious trigger hypothesis. Clin. Vaccine Immunol., 15: 320-326.

Semret, M., D.C. Alexander, C.Y. Turenne, P. de Haas and P. Overduin *et al*., 2005. Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. J. Clin. Microbiol., 43: 3704-3712.

Sevilla, I., S.V. Singh, J.M. Garrido, G. Aduriz, S. Rodriguez, M.V. Geijo *et al*., 2005. Molecular typing of *Mycobacterium avium* subspecies *paratuberculosis* strains from different hosts and regions. Rev. Sci. Tech., 24: 1061-1066.

Shankar, H., S.V. Singh, P.K. Singh, A.V. Singh, J.S. Sohal and R.J. Greenstein, 2010. Presence, characterization and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk and milk products in India by culture, PCR and PCR-REA methods. Int. J. Infect. Dis., 14: 121-126.

Singh, N., S.V. Singh, V.K. Gupta, V.D. Sharma, R.K. Sharma and V.M. Katoch, 1996. Isolation and identification of *Mycobacterium paratuberculosis* from naturally infected goatherds in India. Indian J. Vet. Path., 20: 104-108.

Singh, N., V.S. Vihan, S.V. Singh and V.K. Gupta, 1998. Prevalence of Johne's disease in organized goat herds. Indian J. Anim. Sci., 68: 41-42.

Singh, S.V., A.V. Singh, P.K. Singh, J.S. Sohal and N.P. Singh, 2007. Evaluation of an indigenous ELISA for diagnosis of Johne's disease and its comparison with commercial kits. Indian J. Microbiol., 47: 251-258.

Singh, A.V., S.V. Singh, G.K. Makharia, P.K. Singh and J.S. Sohal, 2008. Presence and characterization of *Mycobacterium avium* subspecies *Paratuberculosis* from clinical and suspected cases of Crohn's disease and in the healthy human population in India. Int. J. Infect. Dis., 12: 190-197.

Singh, A.V., S.V. Singh, J.S. Sohal and P.K. Singh, 2009a. Comparative potential of modified indigenous, indigenous and commercial ELISA kits for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* in goat and sheep. Indian J. Exp. Biol., 47: 379-382.

Singh, S.V., J.S. Sohal, P.K. Singh and A.V. Singh, 2009b. Genotype Profiles of *Mycobacterium avium* subspecies *Paratuberculosis* isolates recovered from animals, commercial milk and human beings in North India. Int. J. Infect. Dis., 13: e221-e227.

Singh, A.V., S.V. Singh, P.K. Singh and J.S. Sohal, 2010a. Genotype diversity in Indian isolates of *Mycobacterium avium* subspecies *paratuberculosis* recovered from domestic and wild ruminants from different agro-climatic regions. Comp. Immunol. Microbiol. Infect. Dis., 33: e127-e131.

Singh, P.K., S.V. Singh, H. Kumar, J.S. Sohal and A.V. Singh, 2010b. Diagnostic application of IS*900* PCR using blood as a source sample for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in early and subclinical cases of caprine *paratuberculosis*. Vet. Med. Int., 10.4061/2010/748621

Singh, A.V., S.V. Singh, D.K. Verma, R. Yadav, P.K. Singh and J.S. Sohal, 2011a. Evaluation of indigenous absorbed ELISA Kit for the estimation of seroprevalence of *mycobacterium avium* subspecies *paratuberculosis* antibodies in human beings in North India. ISRN Vet. Sci., 10.5402/2011/636038

Singh, A.V., S.V. Singh, P.K. Singh, J.S. Sohal and M.K. Singh, 2011b. High prevalence of Mycobacterium avium subspecies *paratuberculosis* ('Indian bison type') in animal attendants suffering from gastrointestinal complaints who work with goat herds endemic for Johne's disease in India. Int. J. Infect. Dis., 15: e677-e683.

Singh, S.V., A.V. Singh, P.K. Singh, A. Kumar and B. Singh, 2011c. Molecular identification and characterization of *Mycobacterium avium* subspecies *paratuberculosis* in free living non-human primate (*Rhesus macaques*) from North India. Comp. Immunol. Microbiol. Infect. Dis., 34: 267-271.

Singh, A.V., D.S. Chauhan, A. Kumar, P.K. Singh and S.V. Singh, 2012a. Potential etiologic link and association between *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease in humans. Res. Rev. J. Immunol., 2: 20-33.

Singh, S.V., A. Tiwari, A.V. Singh, P.K. Singh and B. Singh *et al*., 2012b. Contamination of natural resources (soil and river water) with *Mycobacterium avium* subspecies *Paratuberculosis* in three districts of Uttar Pradesh: A pilot study. Haryana Vet., 51: 1-5.

Singh, S.V., A.V. Singh, S. Gupta, A.S. Rajindran and N. Swain *et al*., 2012c. Interspecies sharing of indian bison type a novel predominant genotype of *Mycobacterium avium* subspecies *paratuberculosis* between naturally infected and endemic flocks of bharat merino sheep and a colony of rabbits (*Oryctolagus cuniculus*) raised on the same ecosystem in South India. Res. Rev. J. Life Sci., 2: 1-8.

Singh, S.V., N. Kumar, S.N. Singh, T. Bhattacharya and J.S. Sohal *et al*., 2013. Genome sequence of the Indian bison type biotype of *Mycobacterium avium* subsp. *paratuberculosis* strain S5. Genome Announc. 1:e00005-13.

Sishodia, A.S., A. Panwar, S.V. Singh, P.K. Singh and A.V. Singh *et al*., 2009. Prevalence of *Mycobacterium avium* subspecies *paratuberculosis*, an animal pathogen, in the population of animal keepers of Ghaziabad and Saharanpur districts of North India using multiple diagnostic tests. Indian J. Comp. Microbiol. Immunol. Infect. Dis., 30: 42-44.

Vilagut, L., A. Pares, O. Vinas, J. Vila, J. De Anta, T. Ma and J. Rodes, 1997. Antibodies to mycobacterial 65-kD heat shock protein cross-react with the main mitochondrial antigens in patients with primary biliary cirrhosis. Eur. J. Clin. Invest., 27: 667-672.

Whan, L., H.J. Ball, I.R. Grant and M.T. Rowe, 2005. Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in untreated water in Northern Ireland. Applied Environ. Microbiol., 71: 7107-7112.

Whipple, D.L., D.R. Callihan and J.L. Jarnagin, 1991. Cultivation of *Mycobacterium paratuberculosis* from bovine fecal specimens and a suggested standardized procedure. J. Vet. Diagn. Invest., 3: 368-373.

Whittington, R.J., I.B. Marsh and L.A. Reddacliff, 2005. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Applied Environ. Microbiol., 71: 5304-5308.

Whittington, R.J., I.B. Marsh and R.H. Whitlock, 2001. Typing of IS *1311*polymorphisms confirms that bison (*Bison bison*) with *paratuberculosis* in Montana are infected with a strain of *Mycobacterium avium* subsp. *paratuberculosis* distinct from that occurring in cattle and other domesticated livestock. Mol. Cell. Probes, 15: 139-145.

Yokota, S., K. Tsubaki, T. Kuriyama, H. Shimizu and M. Ibe *et al*., 1993. Presence in Kawasaki disease of antibodies to mycobacterial heat-shock protein HSP65 and autoantibodies to epitopes of human HSP65 cognate antigen. Clin. Immunol. Immunopathol., 67: 163-170.