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Research Article

CONTAMINATION OF NATURAL RESOURCES (SOIL AND RIVER WATER) WITH MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN THREE DISTRICTS OF UTTAR PRADESH: A PILOT STUDY

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ABSTRACT

The present study investigated the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) bacilli in natural resources (soil and water). A total of 71 samples from the natural resources (51 soil and 20 river water) collected from three districts (Etawah, Agra and Mathura) of South Uttar Pradesh were screened using microscopy and direct IS900 PCR. Positive soil and river water samples were further genotyped using IS1311 polymerase chain reaction-restriction enzyme analysis (PCR–REA) method. Of the 71 samples screened, 33 (46.4%) and 17 (23.9%) were positive for MAP by microscopy and direct IS900 PCR, respectively. Of the 51 soil samples, 27 (52.9%) and 15 (29.4%) were positive for MAP by microscopy and IS900 PCR, respectively. Prevalence of MAP was higher in soil samples of Etawah district followed by Agra and Mathura districts. Of the 20 water samples, 6 (30.0%) and 2 (20.0%) were positive for MAP by microscopy and direct IS900 PCR, respectively. Prevalence of MAP was higher in water samples from Mathura (Yamuna river) district as compared to Agra (Yamuna river). None of the water sample was positive from Etawah district (Chambal river). IS900 PCR positive DNA samples were further genotyped as 'Indian Bison type' by IS1311 PCR-REA. The study recorded microbiological contamination of natural resources and also with MAP in North India. It is therefore, important to restrict contamination of natural resources through animal and human excreta and wastes in order to reduce transmission to animal and human population of the country.

Key words: Environment, soil, water, *Mycbacterium avium* subsp *paratuberculosis*

India is second in human population and first in animal population in the world. In view of the high population density and non-processing of animal and human waste, precious and slowly depleting national natural resources are getting contaminated with highly resistant microbes and provide environment for maintenance and transmission of pathogens to livestock and human populations alike. *Mycobacterium avium* subsp *paratuberculosis* (MAP) causes paratuberculosis or Johne's disease (JD) in domestic and wild ruminants (Clarke, 1997; Olsen *et al.,* 2002) and Crohn's disease (CD) in human beings (Hermon-Taylor, 2009; Pierce, 2009). JD had devastating effect on the livestock productivity and results in huge economic losses (Harris and Barletta, 2001). Clinically and sub-clinically infected animals excrete huge quantities of MAP in feces, milk etc., and within herds transmission occurs either directly, in-utero or by fecal-oral route. MAP are known to have long survival time (more than 1 year) with persistence under adverse environmental conditions (Sung and Collins, 2000; Collins *et al.,* 2001; Whittington *et al.,* 2004) and have the potential of transmission to new host by ingestion, inhalation and inoculation of the bacilli (Pickup *et al*., 2005).

Previous studies from England, France, and U.S. had reported that soil types and soil composition are connected with the incidence of JD in livestock herds (Berghaus *et al.,* 2006; Lombard *et al.,* 2006). In India, MAP infection is endemic in livestock herds and high prevalence of MAP has been reported in domestic livestock, wild ruminants including free living primates (Singh *et al.,* 2010; Singh *et al.,* 2011a). Recently, Singh *et al.* (2008) and Singh *et al.* (2011b) reported high presence of MAP in CD patients, animal attendants, and healthy persons. Based on IS1311 PCR-REA, 'Indian Bison type' has been reported as the new and major genotype in India, infecting multiple host species including human beings (Singh *et al.,* 2009;

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Sohal *et al.,* 2010). Control strategies will require complete information about the survival and distribution of MAP in different hosts, geographical region, environment and natural resources. This study investigates the presence of MAP in the natural resources (soil and river water) in three districts of Uttar Pradesh (UP).

MATERIALS AND METHODS

Collection of Environmental Samples: Fifty one soil samples were collected from Mathura, Agra and Etawah districts of UP (Table 1). Of the 26 soil samples from Mathura district, 13 were from geographical area endemic for JD in goat flocks (Singh *et al.,* 1998; Kumar *et al.,* 2007). The remaining 13 samples from Agra and Etawah district were collected from the pasture lands shared by domestic and wild ruminants.

Twenty water samples were collected from the bank of two major rivers (Yamuna and Chambal) flowing through three districts (Agra, Mathura and Etawah) of UP. Of these, 13 (8 from Vrindavan, Mathura and 5 from Agra) were from Yamuna river during summer season in 2009 and the remaining seven were collected from bank of Chambal river in Etawah district.

Processing of Soil and Water Samples: Soil and water samples were concentrated by centrifugation and stained with Ziehl-Neelsen (ZN) stain. Briefly, approx. 2 gm of soil sample was finely ground in a sterilized pestle and mortar in sterilized DW (10-12 ml). Ground material (15 ml) was centrifuged at 1557 x g for 1 hr at room temperature (RT). Supernatant was discarded and middle layer was decontaminated in 25 ml. of 0.9% hexadecyl pyridinium chloride (HPC) for 18-24 hours at RT and used for making smears. After decontamination and sedimentation, the supernatant was removed and discarded slowly and ≈1ml of sediment left after decontaminant was used for making smears and PCR applications.

Table 1
Screening of soil samples for the presence of *Mycobacterium avium* subsp *paratuberculosis*

Districts	Sample type	Number tested	Number positive microscopic examination (%)	Number positive IS900 PCR (%)
Mathura	Animal shed	13	8 (61.5)	7 (53.8)
	Pasture	13	2 (15.3)	1 (7.6)
	Subtotal	26	10 (38.4)	8 (30.7)
Agra	Pasture	8	5 (62.5)	2 (25.0)
Etawah	Pasture	17	12 (70.5)	5 (29.4)
	Total	51	27 (52.9)	15 (29.4)

DNA Isolation and IS900 PCR: Positive samples in microscopy (soil and water) were processed for the isolation of DNA as per the method of Singh *et al.* (2007) with modifications accordingly. DNA was amplified by PCR using IS900 primers (Vary *et al*., 1990) as per condition described by Kumar *et al.* (2007). The presence of specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis. Positive (MAP 'Indian Bison type') and negative (sterile liquipure water) controls were also run to check for non-specific reactions.

IS1311 PCR-REA: PCR amplification of IS1311 sequence was carried out using M56 and M119 primers as per Sevilla *et al.* (2005). Amplicon sizes of 608 bp were considered positive in IS1311 PCR, after separation on 2% agarose gel stained with ethidium bromide. Restriction endonuclease analysis of IS1311 PCR product was carried out using *Hinf* I and *Mse*1 enzymes as described by Sevilla *et al.* (2005). Band patterns were visualized after electrophoresis on 4% agarose gel and staining with ethidium bromide. Genotype profiles were interpreted as per Sevilla *et al.* (2005).

RESULTS AND DISCUSSION

Mycobacterium avium subsp *paratuberculosis* has the super survival mechanisms and resistance to many physical and chemical agents (Sung and Collins, 2000; Collins *et al.,* 2001; Whittington *et al.,* 2004). Information about the presence of MAP in natural resources is necessary to understand the epidemiology of MAP infection and to formulate control strategies. This study reports the presence of MAP in natural resources (soil and river water) in India. Of the 71 environmental samples screened for the presence of MAP, 46.4% and 23.9% were positive by microscopy and direct IS900 PCR, respectively. Previous studies also reported the persistent survival of MAP in soil, ponds, tap water and pasture from developed countries (Olsen *et al.,* 1985; Whan *et al.,* 2005). Prevalence of MAP was higher in soil samples (microscopic

examination-52.9%, IS900 PCR-29.4%, Table 1) as compared to water samples (microscopic examination-30.0%, IS900 PCR-20.0%, Table 2). The lower positivity of samples in PCR as compare to microscopy may either be due to low count of acid fast bacilli (+1) in samples, therefore during DNA isolation the minute quantities of DNA might have been lost during the process, presence of acid fast bacilli other than MAP or presence of PCR inhibitors in soil and water samples. Higher presence of MAP in soil samples in microscopy indicated moderate to heavy contamination of animal sheds and pasture land with MAP bacilli. In India, domestic and wild ruminants share the natural resources and grazing lands and there is a risk of interspecies transmission of MAP through pasture land (Sohal *et al.,* 2009; Kumar *et al.,* 2010).

Region wise, presence of MAP (Table 1) was higher in soil samples from Etawah (70.5%) followed by Agra (62.5%) and Mathura (38.4%) districts which may be due to the sharing of pasture land by higher population of animal species. The samples from Mathura district were collected from animal shed endemic for MAP infection (Singh *et al.,* 1998; Kumar *et al.,* 2007) and pasture lands. Higher presence of MAP in animal sheds (61.5%) as compared to pasture lands (15.3%) in Mathura district may be due to heavy excretion of MAP in feces of JD-affected animals. Presence of MAP in Agra and Etawah districts was near to the presence of MAP in the sheds of herds endemic for MAP in Mathura district. Higher level of MAP contamination and exposure to clinical cases of JD has been reported as the risk of acquiring MAP infection in human beings (Singh *et al.,* 2008; Singh *et al.,* 2011b). Therefore there is an urgent need to study the prevalence of MAP at the national level and formulate the policies for restriction of spread and interspecies transmission of MAP.

Of the 20 water samples from two rivers (Yamuna and Chambal), 30.0 and 10.0% were positive for MAP using microscopic examination and direct IS900 PCR, respectively (Fig. 1, Table 2). On basis of visual examination, water samples from Yamuna river were yellowish in colour and turbid in nature, whereas water samples from Chambal river were transparent at the time of sample collection. Previous studies also

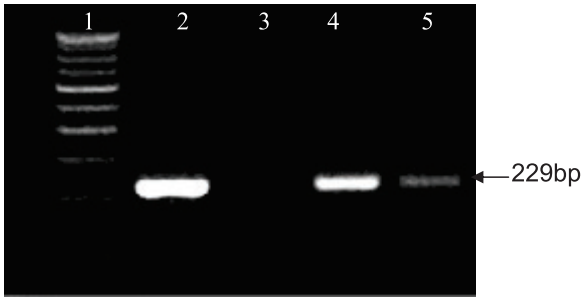

Fig 1. IS900 PCR amplicons for *Mycobacterium avium* subsp *paratuberculosis*. Lane 1= 1Kb (100bp) DNA ladder; Lane 2= Positive control; Lane 3= Negative control; Lane 4= Y3 and Lane 5= Y7.

reported the presence of MAP in lake catchments and river water (Pickup *et al.,* 2006). All the positive samples belonged to Yamuna river and none of the samples was positive from Chambal river. This may be due to the heavy load of sewage inputs in Yamuna river. Absence of MAP in Chambal samples does not mean that Chambal river is free from MAP contamination, this may be due to low level of MAP bacilli in the samples, therefore, repeated and large sample size required for confirmation. However, Chambal river flows mostly through rural and un-habited areas as compared to Yamuna river. MAP is able to survive for about 6 to 18 months in tap or pond water in sealed bottles and for about 15 months in distilled water (Collins *et al.,* 2001). Yamuna water is used both for irrigation and human and animal consumption, therefore MAP infection may be transmitted to large population of animals and human beings through consumption of water or using contaminated vegetables grown in river bed.

Human population is dependent on Yamuna water for domestic water supply in many metropolitans in the country. MAP can survive under chlorine-based disinfectants (Manning and Collins, 2001) and able to

Table 2
Screening of water samples for the presence of *Mycobacterium avium* subsp *paratuberculosis*

River	Place	No. of ghats/ sample	Number positives by	
			Microscopic examination (%)	IS900 PCR (%)
Yamuna	Vrindavan, Mathura	8	4 (50.0)	1 (12.5)
	Agra	5	2 (40.0)	1 (20.0)
	Subtotal	13	6 (46.1)	2 (15.3)
Chambal	Etawah	7	0 (0.0)	0 (0.0)
	Total	20	6 (30.0)	2 (10.0)

survive within protozoa that are usually bacteriovores and inside protozones it may acquire a more pathogenic phenotype for human beings (Hermon-Taylor, 2000). Previous study has reported the relationship between the river and city wards with significantly increased incidences of CD, with the transmission of infection to humans by inhalation of MAP-laden aerosols from the river (Pickup *et al.,* 2005). Therefore, presence of MAP in Yamuna water may pose higher risk of acquiring MAP infection to dependent human and animal populations.

Genotyping of MAP bacilli revealed the presence of 'Indian Bison type' genotype of MAP in soil and water samples (Fig 2). 'Indian Bison type' is the major genotype of MAP, infecting domestic and wild ruminants including human beings in India (Singh *et al.,* 2009; Sohal *et al.,* 2010). Previous studies reported the interspecies transmission of 'Indian Bison type' genotype (Singh *et al.,* 2009; Sohal *et al.,* 2009). The present study indicated that interspecies transmission of 'Indian Bison type' genotype in different livestock species and human beings may be due to the sharing of soil, pasture and water resources. Survival of MAP in the manure and soil increases the opportunities for contact between MAP and animals and thus enhances the chances for contamination of surface water by rainfall run-off. Future research should be focused towards deciphering the mechanisms behind the ability of MAP to survive and grow under suboptimal conditions for the control of disease in animal hosts and restrict the spread of bacilli in environment to minimize the risk of human exposure.

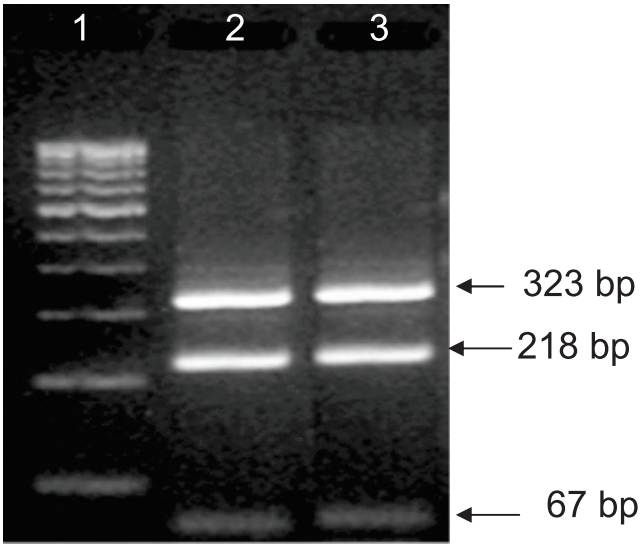

Fig 2. IS1311 PCR-REA analysis of IS900 positive *Mycobacterium avium* subsp *paratuberculosis* DNA. Lane 1= 1Kb (100bp) DNA ladder; Lane 2= Y3 ('Indian Bison type') and Lane 3= Y7 ('Indian Bison Type')

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