



Research Article

Prevalence and genotypes of *Mycobacterium avium* subspecies *paratuberculosis* in large ruminants of Eastern Uttar Pradesh, North India

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Abstract: Uttar Pradesh is the fourth largest, most populous and leading milk and meat producing state in India. Despite the huge livestock population, information on the status of paratuberculosis homogeneity and heterogeneity of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) isolates of eastern Uttar Pradesh is non-existent. Present study was aimed to estimate the presence of MAP in large ruminants (Cattle and Buffaloes) of eastern Uttar Pradesh. A total 108 fecal samples were collected from farmer's herds of large ruminants (cattle and buffaloes) from different geographical regions (Chandauli, Mughalsarai, Gazipur, and Naugarh) of eastern Uttar Pradesh and screened for the presence of MAP infection using microscopic examination, direct IS900 PCR and culture on Herrold egg yolk (HEY) medium. The isolates recovered on HEY medium were subjected to molecular identification and genotyping using IS900 PCR and IS1311 PCR-REA method, respectively. Of the 108 fecal samples, 25 (23.14%) and 11 (10.18%) samples were positive for the presence of acid-fast bacilli and growth on HEY medium, respectively. Species-wise, 17.5, 7.5% and 26.5, 11.7% fecal samples from cattle and buffaloes were found positive for the presence of acid-fast bacilli and growth on HEY medium, respectively. Isolates recovered on HEY medium with mycobactin J were positive for IS900 sequence and genotyped as Bison Type using IS1311 PCR-REA method. Present study is the first report on the presence of MAP infection and 'Bison Type' genotype of MAP in eastern Uttar Pradesh. These findings will be useful for the intervention of effective control measures in order to reduce the prevalence of MAP infection in domestic livestock species and prevent its spread to the human population in the regions.

Keywords: Paratuberculosis, Domestic large ruminants, *Mycobacterium avium* subspecies *paratuberculosis*, Bison Type, Eastern Uttar Pradesh.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a well-known cause of chronic, infectious incurable inflammatory condition known as paratuberculosis or Johne's disease in domestic animals (cattle, goat, sheep, buffalo, camel and yak etc.), free ranging animals (rabbit, fox, weasels, stoat etc.), wildlife species (antelopes, blue bulls, deer, elk, bison, llamas, bighorn sheep and alpacas) including non-human primates [1-3]. MAP has also been associated with the Crohn's disease in humans [4,5]. MAP infection in animal results in progressive weight loss, weakness with or without chronic diarrhea, debilitation, and emaciation. Paratuberculosis drastically reduced the

productivity of domestic animal herds by reduced milk production, poor feed conversion, increased susceptibility to mastitis, reduced reproductive efficiency, premature culling, and reduced slaughter values, and cause significant economic losses to the livestock producer worldwide [6].

India has highest livestock population (512.05 million) and one of the leading countries in milk and meat production in the world. Recent studies have been reported high prevalence of paratuberculosis (~23.0%) and 'Indian Bison Type' genotype of MAP in domestic livestock species of different parts of the country [7-9]. The available information about the burden and genotypes of MAP in domestic livestock species is sparse and is in the form of case series or observational

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studies. The national prevalence of paratuberculosis and associated economic losses to the livestock production system has not been estimated in India. Recently, Rawat *et al.*, [10] has recorded high economic losses (Rs. 16,87,977.5) due to the outbreak of paratuberculosis in a commercial dairy farm of 79 Holstein-Friesian cattle in Rajasthan, North India. It has been thought that paratuberculosis is endemic in domestic animal herds of every state in the country [11].

Among the states, Uttar Pradesh has the largest livestock (61 million) and human (215 million) population in India. It shares 17.6% of total milk and 19.1% of total meat production in the country [12]. Despite the huge livestock population and production, information on the status of MAP infection in animals is only available from western regions of Uttar Pradesh [7,13,14]. Information on the status of paratuberculosis and homogeneity & heterogeneity of MAP isolates of eastern Uttar Pradesh is not available and required to understand transmission dynamics of disease and formulation of effective control strategies. This study was aimed to investigate the presence of MAP and their genotypes in large ruminants (cattle and buffaloes) from eastern Uttar Pradesh.

2. Materials and Methods

Present study was conducted at Department of Microbiology and Molecular Biology, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Taj Ganj, Agra, UP, India during the period of 2015-2016. The work has been approved by Institute Animal Ethics Committee.

2.1 Animals and collection of clinical sample

A total of 108 fecal samples were collected from domestic large ruminants (cattle -40 and buffaloes -68) of eastern Uttar Pradesh for the screening of MAP infection. All the animals belonged to farmer's herds (average size-6 animals) of 608 animals. Of the 108 fecal samples, 32, 48, 24 and 4 fecal samples belonged to Chandauli, Naugarh, Gazipur and Mughalsarai regions of eastern Uttar Pradesh, respectively. The demographic information and health status of the animals were recorded during the period of sample collection. Of the 108 animals, 13 were calf (below 2 years) and 95 animals were adults. Sex-wise, all the animals were female. Fecal samples were directly taken from the rectum with the help of index finger in sterile poly bags. Finger used was washed every time for collection of fecal sample from each animal. Slip with animal number was enclosed and bags were sealed with tape to avoid drying the material during transport and storage in refrigerator at 4°C. The fecal samples were processed for the detection of MAP using microscopic examination (Ziehl-Neelsen staining), culture on Herrold's Egg Yolk (HEY) medium and direct IS900 PCR.

2.2 Microscopic examination (Ziehl-Neelsen staining)

Approximately, 2 gram of fecal sample was finely grounded in sterilized pestle and mortar, with the help of sterilized D.W. (10-12ml). Grounded material was transferred to 15ml centrifuge tubes. Tubes were centrifuged at 1557 x g for 1 hour at room temperature. Supernatant was discarded and middle layer was decontaminated in 25ml of 0.9% Hexadecylpyridinium Chloride (HPC) for 18-24 hours at room temperature. After decontamination and sedimentation, the supernatant was removed slowly and of ~1ml of sediment left, 0.2ml used to prepare the smear and stored at -20°C for the processing of direct IS900 PCR.

Smears were prepared using residual sediment on clean slides and air dried. Once completely dry, smears were quickly heat fixed over open flame and cooled at room temperature. Slides were flooded with carbolfuchsin and heated gently till steaming and were left for 05 minutes. Slides were then rinsed in water. Slides were then destained in acid alcohol for 30 seconds and repeated the step till pink colour stop coming followed by washing with water. Slides were then counterstained with methylene blue for 30 sec, before a final washing with water. Glass slides were gently blotted and air dried prior to screening by microscopic. Slides displaying pink coloured short rods indistinguishable to MAP were considered positive.

2.3 Culture on Herrold's Egg Yolk (HEY) medium

After decontamination, the supernatant was removed slowly and from 1ml sediment about 0.2ml of sediment was inoculated on HEYM slants (with and without mycobactin J). The inoculated slants were kept at 37°C in incubator first in slanting position for 3 to 5 days and when moisture had evaporated from slants, tubes were incubated in vertical position in BOD incubator. Slants were observed for any growth at weekly interval up to 30 weeks. Contaminated slants were discarded and colonies appearing around or later than 6-8 weeks of inoculation resembling that of MAP were selected for further identification.

2.4 Direct IS900 PCR

The remaining decontaminated fecal material was processed for the isolation of DNA and direct IS900 PCR using the MAP-specific primers described by Vary *et al.*, [15]. The PCR product of 229bp was considered as positive for MAP.

2.5 Molecular Identification of MAP isolates by colony PCR

All the isolates recovered on HEY medium with mycobactin J were subjected to DNA isolation and molecular identification using IS900 PCR as per the method described by Singh *et al.*, [7]. Positive (MAP 'Indian Bison type') and negative (sterilized liquipure water) controls were also run simultaneously.

2.6 Genotyping of MAP isolates

All the IS900 positive MAP isolates were subjected to IS1311 PCR using the previously described primers [16]. After separation on 2% agarose gel and staining with ethidium bromide, 608bp amplicon was considered positive in IS1311 PCR. IS1311 PCR-REA was carried out as per Singh *et al.*, [7] and genotype profiles were interpreted as per Whittington *et al.*, [17].

3. Results

3.1 Screening of fecal samples using Microscopic examination, culture on HEY medium and Direct IS900 PCR

Of the 108 fecal samples, 25 (23.14%), 13 (12.03%) and 14 (12.96%) samples were found positive for the presence of acid-fast bacilli morphologically indistinguishable to MAP (Fig. 1) using microscopic examination (ZN staining), culture and direct IS900 PCR, respectively. The region-wise prevalence of MAP in cattle and buffaloes was found to be quite variable (Table 1) Species-wise, the prevalence of MAP was lower in cattle (Microscopic examination- 17.5%, Culture- 12.5% and direct PCR- 10.0%) as compared to Buffalo (Microscopic examination- 26.4%, Culture- 11.7% and direct PCR- 16.7%) samples (Table 1).

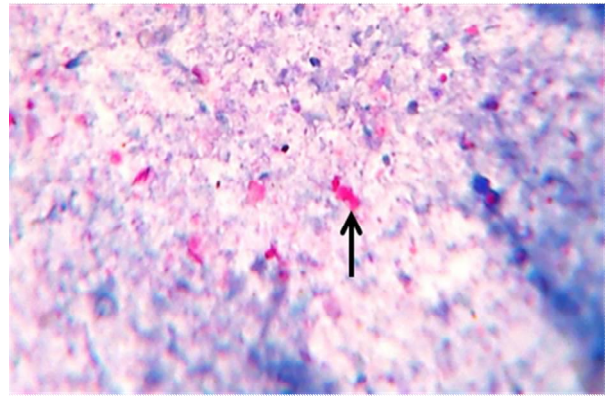


Fig. 1. Detection of Acid-fast bacilli (pink color) in fecal samples of domestic livestock using microscopic examination (100X).

3.2 Molecular identification and genotyping of isolates

Of the 13 isolates recovered on HEY medium with mycobactin J, good quality DNA was obtained from 7 (53.8%) samples. All the 7 DNA samples were found positive for the presence of IS900 sequence and identified as MAP. All the 7 MAP isolates were genotyped as 'Bison Type' using IS1311 PCR-REA method (Fig. 2).

Table 1. Screening of fecal samples of large ruminants (cattle and buffalo) of eastern Uttar Pradesh for the presence of MAP using microscopic examination and culture on HEY medium and direct IS900 PCR.

Species	Region	No. of Samples	No of positives (%)		
			Microscopy	Culture on HEY medium	Direct IS900 PCR
Cattle	Chandauli	13	3	1	2
	Naugarh	22	2	4	1
	Mughalsarai	03	2	0	0
	Gazipur	02	0	0	0
	Sub-total	40	7 (17.5%)	5 (12.5%)	3 (7.5%)
Buffaloes	Chandauli	19	6	0	6
	Naugarh	26	6	8	5
	Mughalsarai	1	0	0	0
	Gazipur	22	6	0	0
	Sub-total	68	18 (26.4%)	8 (11.7%)	11 (16.17%)
Grand Total	108	25 (23.1%)	13 (12.03%)	14 (12.96%)	

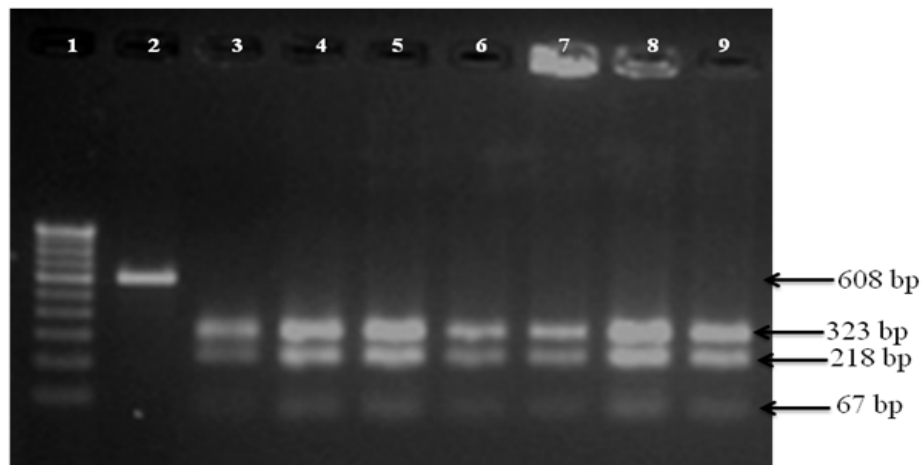


Fig. 2. Genotyping of IS900 positive MAP isolates using IS1311 PCR-REA method (Lane 1: 100bp ladder, Lane 2: Undigested PCR product of IS1311 PCR, Lane 3-9: test samples).

4. Discussion

Present study was aimed to investigate the status of MAP infection and their genotypes in cattle and buffalo population of eastern Uttar Pradesh. The fecal samples collected from cattle and buffaloes of different geographical regions (Chandauli, Naugarh, Mughalsarai, and Gazipur) of eastern Uttar Pradesh were screened for the presence of MAP using microscopic examination, culture, and direct IS900 PCR. Previously, various researchers have used microscopy examination, fecal culture and direct PCR as diagnostic tools for the detection of MAP infection in domestic animals [7,18,19] and wild animals [20,21].

In the present study, of the 108 fecal samples, 25 (23.14%), 13 (12.03%) and 14 (12.96%) samples were found positive for the presence MAP using microscopic examination, culture, and IS900 PCR, respectively. The high presence of acid-fast bacilli in fecal samples using microscopic examination indicated the presence of mycobacteria other than MAP in fecal samples of large ruminants of eastern Uttar Pradesh. Previous studies have also reported the presence of mycobacteria other than MAP in fecal samples of large ruminants of western Uttar Pradesh [22,23].

In the present study, HEY medium with mycobactin J was used for the isolation of MAP. Previous studies have been reported that culture of MAP from clinical samples is the 'gold standard' test for the diagnosis of MAP infection [24]. Of the 108 fecal samples, 13 (13.88%) samples were found positive for growth on HEY medium with mycobactin J. The prevalence of MAP was slightly higher in cattle (12.5%) as compared to Buffalo (11.7%) population of eastern Uttar Pradesh. Previous studies have been reported high presence of MAP in cattle and buffalo population of different regions of western Uttar Pradesh, North India using multiple diagnostic tests [7,8,25,26]. Mishra *et al.*, [18] screened the fecal samples from three dairy cattle herds located in Mathura district of Western Uttar Pradesh for the presence of MAP and found 28.3 and 20.8% animals as positive using culture and ELISA test, respectively. Sharma *et al.*, [25] investigated the presence of MAP in lactating Indian dairy cattle using three diagnostic tests (milk culture, milk-ELISA and milk-PCR) and reported 84.0%, 32.1% and 6.0% animals as positive for MAP using milk-culture, m-ELISA and m-IS900 PCR, respectively. Recently, Singh *et al.*, [8] studied the 'Bio-load' of MAP in the domestic livestock population of India and reported 39.3% 'Bio-load' of MAP in cattle in the country. Singh *et al.*, [7] screened 326 fecal samples of buffaloes of different geographical regions (Agra, Mathura, Bareilly and Ludhiana region) of North India and reported 31.7% animals as positive for the presence of MAP infection using bacterial culture test. In Agra region of North India, Yadav *et al.*, [26]

screened 50 tissues of sacrificed buffaloes using culture method and reported the presence of MAP in tissues of 48.0% buffaloes. Using indigenous ELISA kit as diagnostic test, Singh *et al.*, [13] screened a serum sample indigenous ELISA and reported 28.6% buffalo as positive for MAP infection in Northern India. Findings of the present study indicated that the prevalence of MAP infection in large ruminants (cattle and buffaloes) of eastern Uttar Pradesh is slightly lower as compared to other parts of the country. This can be attributed to the geographical variation or screening of animals of farmers herds in the present study.

In the present study, IS900 PCR method was used for the molecular identification of MAP isolates. Previous studies have also been used IS900 PCR as diagnostic tool for the identification of MAP isolates from domestic ruminants [7,25,26]. The genotypes of MAP were studied by IS1311 PCR-REA typing method of Whittington *et al.*, [18]. This technique has been frequently used by various researchers for the characterization of MAP isolates and to study the transmission dynamics of MAP infection in different livestock population [7,16,18]. In the present study, all the IS900 positive isolates were genotyped as 'Bison Type' genotype of MAP using IS1311 PCR-REA method. This finding supports previous studies which reported 'Bison type' genotype as the most predominant genotype of MAP infecting livestock population in India [7,8,25,26].

5. Conclusion

Present study first time reported the status of MAP infection and presence of 'Bison type' genotype of MAP in domestic livestock population of eastern Uttar Pradesh. The major limitation of the present study is the small sample size and therefore, it is not representative of huge livestock population of various regions of eastern Uttar Pradesh. In fact, this limitation was observed in most previous studies on the presence of MAP in human and animals of North India. Nationwide and Statewide representative data on the prevalence of MAP infection in domestic animals are needed to be established to formulate effective national control program for paratuberculosis in the country. The findings of this study are quite reassuring in presence of paratuberculosis and 'Bison type' genotype of MAP in domestic livestock of eastern Uttar Pradesh.

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