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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

A.V. Singh [a], S.V. Singh [a,*], G.K. Makharia [b], P.K. Singh [a], J.S. Sohal [a]

[a] *Veterinary Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom, Farah, Mathura (UP), 281 122 India*
[b] *Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India*

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IS900 PCR;
ELISA

Summary
Objectives: To investigate and characterize *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in patients with Crohn's disease, attendants of animals with suspected infection, and healthy humans, using multiple diagnostic tests.
Methods: A total of 119 samples (35 stool, 76 serum, three blood clots, and five biopsies) were collected from five patients with Crohn's disease, eight attendants of animals with Johne's disease, and 93 apparently normal control subjects (Agra region) from North India. Samples were screened for the presence of MAP by smear examination, culture of stool, blood clot and biopsies, and ELISA. Colonies obtained by culture were further characterized using polymerase chain reaction (PCR) with IS900 MAP-specific primers.
Results: Using all diagnostic modalities, MAP and/or MAP antibodies were identified in 100% (5/5) of subjects with Crohn's disease; 75.0% (6/8) of attendants of MAP infected animals were positive and 38.0% (27/71) of apparently normal controls were also positive. Most sensitive test was ELISA (100%, 5/5), followed by culture (80.0%, 4/5), and acid-fast staining. Ziehl–Neelsen staining was positive in 37.5% (3/8) of subjects with active animal husbandry practices. In 71 serum samples from control subjects, seroprevalence of MAP was 38.0% using indigenous protoplasmic antigens (PPA) and 36.6% using commercial PPA. Of the serum samples from the Crohn's disease patients, 100% (5/5) were positive by ELISA using indigenous PPA and 40.0% (2/5) were positive by ELISA using commercial PPA. IS900 PCR was used to characterize tiny colonies of MAP that grew extremely slowly on Herrold's egg yolk medium, and of 15 (42.8%) cultures, 14 (93.3%) were typed as MAP.

* Corresponding author. Tel.: +91 565 2763260x269; fax: +91 565 2763246.
E-mail address: shoorvir.singh@gmail.com (S.V. Singh).

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Conclusions: Paper documented the presence of MAP in all patients with Crohn's disease, in some animal attendants who had the history of working with goat herds infected with Johne's disease and in few normal healthy individuals. Presence of Ziehl Neelsen positive MAP. In the stool of attendants working with MAP-infected animals was unique to humans. ELISA based on antigens derived from indigenous MAP 'bison type' genotype of goat origin was most sensitive modality for screening Crohn's disease patients.

Introduction

In India, (most populous country in the world after China), enteric infections are the cause of major health problems; Crohn's disease was first reported from India in 1986. Although *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is endemic in the ruminant population of this country, the association of MAP with enteritis or Crohn's disease has not been investigated so far.

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are complex disorders reflected by wide variations in clinical practice. IBD has long been considered to affect the populations of North America and Europe more than those of Asia.[1] Crohn's disease was considered almost non-existent in India until 1986.[2] In the last 10 years, however, Crohn's disease has been reported more frequently from different parts of India, especially the south.[3–8] These studies are only tip of the iceberg, since diagnostic facilities are restricted to few metropolitan cities, but the largest risk populations mainly live in villages and small towns. Abdominal ailments are the leading causes of sickness and death in large sections of the 1.2 billion plus human population. Recently very high prevalence of ulcerative colitis has been reported from Northern India.[9] Pain and frequent bouts of diarrhea overlap as clinical signs in most of the cases of abdominal illnesses. Therefore, differential diagnosis between these two diseases before actual treatment starts, is important.

Though there is no consensus on the etiology of Crohn's disease. MAP has been reported from cases of Crohn's disease worldwide.[10–17] Recently, there has been renewed interest in the possible association of MAP and Crohn's disease, largely due to the isolation of genetically identical pathogenic MAP from patients with Crohn's disease.[17] Though, Crohn's disease was first reported in India in 1986, the association of MAP with cases of Crohn's disease has not yet been studied, primarily due to a lack of indigenous diagnostic kits and reagents. MAP is endemic in the ruminant population of the country.[18–23] A pilot scale study was undertaken to estimate the presence and association of MAP and antibodies in clinical cases of Crohn's disease, in suspected cases of IBD in animal attendants, and in the healthy human population, using multiple diagnostic tests.

Materials and methods

The presence of MAP was estimated in chronic cases of Crohn's disease, in suspected (in-contact) cases of IBD in animal attendants, and in healthy persons by smear examination, culture (stool, blood clot, and biopsies), and ELISA (using indigenous soluble PPA from MAP 'bison type' of goat origin and commercial PPA from MAP 'bovine' genotype). MAP cultures were characterized using specific IS900 PCR.

Biopsies and stool and blood samples

A total of 119 samples (35 stool, 76 serum, three blood clots, and five biopsies) were collected from 93 healthy persons, eight animal attendants suffering with long-term colitis (IBD), and five patients with chronic Crohn's disease.

Crohn's disease patients: Samples (stool, blood clots, serum, and biopsies) were collected from five patients diagnosed with chronic Crohn's disease at the Gastroenterology Department of All India Institute for Medical Sciences (AIIMS), New Delhi. These patients were diagnosed on the basis of history, clinical picture, clinical pathology, and colonoscopy. Blood samples were also collected from three patients. These chronic Crohn's disease patients had no history of contact with animals and were undergoing treatment for the Crohn's disease at AIIMS, New Delhi.

In-contact animal attendants with suspected infection: Eight stool samples were obtained from animal attendants who had worked for the past 10—15 years with goatherds endemic for Johne's disease[19,23–27] and were chronic colitis patients (suspected for Crohn's disease on the basis of history, symptoms, and treatment received at human dispensary at Central Institute for Research on Goats (CIRG), Makhdoom. On personal interview they were found to be silently suffering from sub-clinical and clinical symptoms of IBD and/or colitis (weight loss, chronic abdominal pain, frequent bouts of loose motions, reduced appetite, anemia, weakness, tendency to get tired easily). These workers were very poor, illiterate, and could not afford the cost of medical assistance, therefore, they neither continued with treatment nor did seek advance medical interventions. These workers also had their own stock of goats and sheep. Sampling was difficult since they lost hope of getting cured and only stool samples were submitted for screening.

Healthy population: Twenty two stool and eight serum samples were obtained from healthy persons residing in and around CIRG campus, Makhdoom. Of these persons, one had been handling cultures of MAP since 1992 and maintained normal health, while one had history of consuming raw cow's milk for few weeks and was suffering from clinical Crohn's disease (high erythrocyte sedimentation rate, weight loss, repeated bouts of loose motions, anemia, low body weight, unable to take stress, etc.). Sixty three serum samples were randomly collected from healthy persons in Agra city (North India), who were easily convinced to volunteer for the study.

Processing of biopsies. stool and blood samples for isolation of MAP

Approximately 2 g of stool sample was concentrated by centrifugation and stained with Ziehl—Neelsen (ZN) stain,

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Table 1 Screening of stool samples by smear examination, culture, and IS900 PCR for the presence of *Mycobacterium avium* subspecies *paratuberculosis*

Study group	Stool samples	Positive in ZN staining [*n* (%)]	Positive in cultures [*n* (%)]	PCR from MAP cultures [*n* (%)]
Crohn's disease patients[a]	5	0	4 (80.0)	4 (80.0)
Animal attendants with suspected infection[b]	8	3 (37.5)	5 (62.5)	5 (62.5)
Healthy people[c]	22	0	6 (27.2)	5 (22.7)
Total	35	3 (8.5)	15 (42.8)	14 (40.0)

ZN, Ziehl–Neelsen; PCR, polymerase chain reaction; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.
[a] Confirmed cases of Crohn's disease at the All India Institute for Medical Sciences, New Delhi.
[b] Working in goatherds endemic for Johne's disease, and with different degrees of symptoms for colitis or inflammatory bowel disease (chronic stomach pain, constipation, bouts of loose motions, weakness, tiredness, anemia, etc.).
[c] Including one laboratory worker who had been culturing MAP since 1992 and one with a history of raw cow's milk consumption.

MAP was isolated as per the method of Whipple and co-workers[28] with some modifications.[26] Briefly, 2 g of stool or 2 cubic cm of tissue biopsy or 2 g of blood clot was finely ground in a sterilized pestle and mortar and suspended in water. Stool samples were centrifuged and middle layer was further decontaminated. In biopsies and blood clots the suspension in water was allowed to stand for 5–6 hours at room temperature and supernatant was decontaminated in 0.9% hexadecyl pyridinium chloride (HPC) and were then kept undisturbed for 12–18 hours at room temperature. The supernatant was discarded and 0.2 ml of the sediment was inoculated on slants of Herrold's egg yolk medium (three containing mycobactin J and one without mycobactin J). Antibiotics or anti-fungal agents were not added to the medium. Slants were incubated at 37 °C and slants were screened for the appearance of colonies every 15 days.

Enzyme-linked immunosorbent assay (ELISA)

The indigenous ELISA kit initially developed for the screening of goats[27] was standardized for humans. Soluble PPA was prepared from the native 'bison type' genotype of MAP isolated from a terminal case of Johne's disease in a goat.[29] MAP frequently isolated from the goat, sheep, cattle, and buffalo populations of North India have been genotyped as 'bison type' (Whittington RJ, 2001, personal communication).[29] Purified commercial antigen (PPA) of 'bovine' MAP was procured from Allied Monitor, Inc., USA. Antigens from 'bison type' and 'bovine' MAP were standardized at 0.1 μg and 2.0 μg per well of the microtiter plate, respectively. Serum samples were used in 1:50 dilution and anti-human horseradish peroxidase conjugate (Sigma) in 1:8000 dilution. Serum samples from culture-positive Crohn's disease patients and culture-negative healthy persons were used as positive and negative controls, respectively. Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios.[30]

IS900 PCR (Colony PCR)

Positive MAP cultures were processed for DNA isolation as per van Embden et al.[31] and van Soolingen et al.[32] DNA was amplified by PCR using specific IS900 primers.[33] The 229-bp fragment targeting the specific IS900 sequence was amplified from template DNA. Briefly, in a volume of 50 μl of reaction mixture, 1 μl of each primer (forward primer: 150 C 24-mer; reverse primer: 921, 25-mer), 22 μl red dye Master Mix (Taq DNA polymerase, dNTPs, reaction buffer with 1.5 nM magnesium chloride), 24 μl de-ionized water, and 2 μl of template DNA were included (total volume 50 μl). A total of 35 cycles was performed in a thermocycler (MJ Research) for complete amplification reaction. The total time taken for 36 cycles was 1.20 h. The reaction conditions were: initial denaturation at 94 °C for 4 min (1 cycle), denaturation at 94 °C for 10 s, annealing at 61 °C for 10 s, extension at 72 °C for 10 s (35 cycles), and a final extension at 72 °C for 10 min. The presence and yield of the specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis. Positive (MAP 'bison type') and negative (sterile liquipure water) controls were also run to check for contamination.

Results

Smear examinations

Of the smears of stool samples taken from the animal attendants with suspected infection, 37.5% (3/8) were positive for acid-fast bacilli (AFB) indistinguishable from MAP. None of the stool samples from Crohn's disease patients and healthy persons was positive for AFB. Cumulatively, of the 35 stool samples, 8.5% were positive. Of the three positive from the suspected group, two were also positive in culture of stool and one case was detected independently (Tables 1 and 2).

Isolation and characterization of MAP colonies

The majority of MAP colonies grew at around 75 days of incubation on Herrold's egg yolk medium with mycobactin J (Allied Monitor, Inc., USA). Primary colonies were identified on the basis of culture characteristics, bacterial morphology, slow growth, mycobactin J dependency, acid-fastness, and by IS900 PCR.

Table 2 Comparisons of smear examination and culture of stool samples

	Culture positives	Culture negatives
Smear positives[a]	2	1
Smear negatives	13	19

[a] Ziehl–Neelsen staining.

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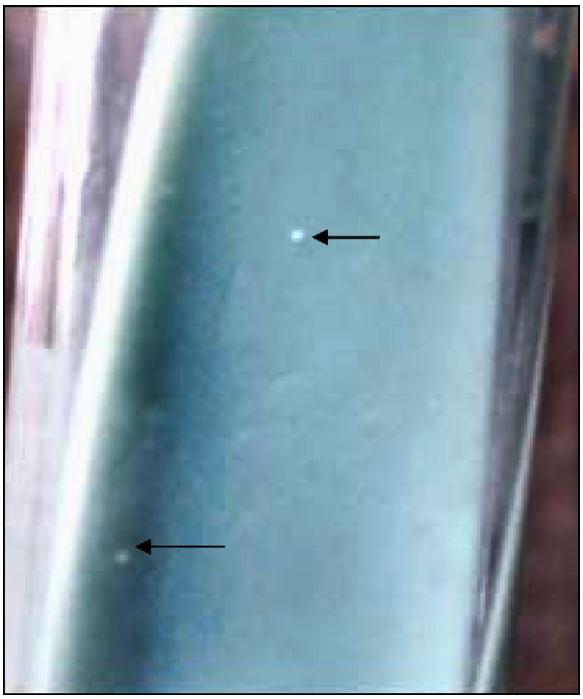

Figure 1 *Mycobacterium avium* subspecies *paratuberculosis* colonies from stool sample of Crohn's disease patient (A35).

Isolation of MAP from stool samples: Of the 35 stool samples screened by culture, 15 (42.9%) were positive for the presence of MAP. (Figure 1). Maximum positives (80.0%) samples were from the Crohn's disease patients, followed by the animal attendants with suspected infection (62.5%) and healthy persons (27.2%) (Table 1). Animal attendants were patients with chronic stomach problems (IBD) and had continuous abdominal pain. In the healthy group, the person handling MAP cultures and the person with a history of raw milk consumption were among those with positive cultures.

Isolation of MAP from biopsies of Crohn's disease patients: of the five Crohn's disease patients, four (80.0%) were positive for MAP in culture of both biopsies and stool samples.

Isolation of MAP from blood clots of Crohn's disease patients: Of the three blood clots, two (66.6%) were positive. These individual were also positive for MAP in stool and biopsy cultures.

Comparison of smear examination and stool culture: Of the 35 stool samples, 8.5% and 42.8% were positive in smear examination and culture, respectively. The 5.7% of samples were positive by two tests and 54.2% of samples were negative in the two tests. The 2.8% and 37.1% of samples were positive independently in smear examination and culture, respectively. Cumulatively, two tests detected 45.7% stool samples as positives (Table 2).

ELISA

All five Crohn's disease patients were positive by ELISA using indigenous PPA, whereas in commercial PPA only two were positives. The indigenous antigen (PPA) had better correlation with culture (stool and biopsies) from Crohn's disease patients (Table 3). In healthy persons and Crohn's disease patients, seroprevalence of MAP was 42.1% [(32/76) using indigenous PPA] and 40.8% [(31/76) using commercial PPA]. Cumulatively, the two antigens detected 57.8% (44/76) of persons as positives (Table 4). Only 25.0% of serum samples were positive by both antigens. The 15.7% of serum samples were positive exclusively by indigenous PPA and 17.1% were positive exclusively by commercial PPA (Table 4).

Polymerase chain reaction (PCR)

Of the 21 cultures (15 stool samples, four biopsies, and two blood clots) processed for DNA isolation and IS900 PCR, 20 (95.2%) were amplified giving the specific 229-bp PCR product (Figure 2). All the cultures from Crohn's disease patients and animal attendants with suspected infection were characterized as MAP. Of the six cultures from healthy persons, five (83.3%) were positive (Table 1).

Discussion

There is increasing evidence that Crohn's disease may have an infectious etiology, and the most plausible candidate is MAP. Intriguingly, Koch's postulates have been fulfilled for MAP and Crohn's disease, though they have not been met for *Mycobacterium leprae* and leprosy.[34] The problems in diagnosing MAP infections in humans are primarily due to difficulties in growing MAP in vitro.[35] Achieving growth in artificial medium may be even more difficult when the organism is

Table 3 Screening of stool and serum samples from Crohn's disease patients[a] by smear examination, culture, IS900 PCR, and ELISA test kit

Lab code	Age (years)	Sex	ZN staining	Stool culture	IS900 PCR	Plate ELISA kit	
						PPA from MAP 'bison type' strain (indigenous)	PPA from MAP 'bovine' strain (commercial)
A35	35	F	–	+	+	+	+
A42	42	M	–	+	+	+	–
A45	35	M	–	+	+	+	–
A49	32	M	–	–	–	+	–
A53	30	M	–	+	+	+	+

ZN, Ziehl–Neelsen; PCR, polymerase chain reaction; PPA, protoplasmic antigens; MAP, *Mycobacterium avium* subspecies *paratuberculosis*; M, male; F, female.
[a] Diagnosed and undergoing treatment at the Gastroenterology Department of the All India Institute of Medical Sciences, New Delhi.

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Table 4 Comparative evaluation of protoplasmic antigens of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) 'bison type' and MAP 'bovine' origin for the sero-diagnosis of antibodies against MAP by ELISA test

	Positives with PPA 'bison type'	Negatives with PPA 'bison type'
Positives in PPA 'bovine' strain	19	12
Negatives in PPA 'bovine' strain	13	32

PPA, protoplasmic antigens.

initially in its cell-wall-deficient form.[35–37] The present study has also revealed that MAP colonies of human origin are highly fastidious and extremely slow growing – only tiny colonies were visible even after prolonged incubation. None of the stool samples from Crohn's disease patients and healthy persons was positive in ZN staining. The presence of ZN negative MAP in Crohn's disease patients is compatible with the theory that MAP in man is present in the cell-wall-deficient form (spheroplasts) frequently reported in the literature.[36,38] Interestingly three of the eight stool samples from the animal attendants working with goatherds endemic for Johne's disease were ZN positive. Prior to this report there was only one report of ZN positive IS900 geno-typed MAP, identified in a hemophiliac man with AIDS.[39] The ZN positive animal attendants with suspected infection may be indicative of recent feco-oral contamination from goats with Johne's disease, or these animal attendants may have fecal–oral contamination in addition to chronic ZN-MAP infection as revealed by symptoms of chronic colitis. ZN positive colonies grew faster than ZN negative colonies.

In culture, 42.8% stool samples were positive for MAP infection. The presence of MAP was highest in Crohn's disease patients, followed by animal attendants with suspected infection and healthy persons. The recovery of MAP from biopsies and stool samples of Crohn's disease patients confirmed the association between MAP and Crohn's disease. The high presence of MAP in animal attendants correlated with the high prevalence of MAP in goatherds.[19,23–25,27] Attendants may have contracted infection from endemic herds. The isolation of MAP from 27.2% of healthy persons may be due to the high presence of MAP in the environment. Of the three persons detected in the healthy group, one had been handling MAP cultures since 1992 and one had a history of raw cow's milk consumption and typical clinical symptoms of Crohn's disease. Fourteen of the 15 positive cultures were IS900 positive by PCR (colony PCR).

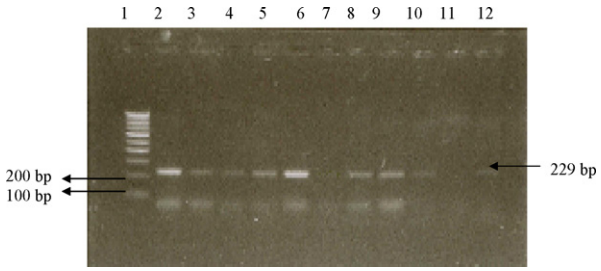

Figure 2 IS900 PCR products obtained with the DNA from stool and biopsy culture positive isolates. Lane 1: marker (100-bp ladder); lane 2: A43 (DNA from Crohn's disease patient biopsy culture); lane 3: A54 (DNA from Crohn's disease patient biopsy culture); lane 4: A38 (DNA from Crohn's disease patient blood clot culture); lane 5: A35 (DNA from Crohn's disease patient stool culture); lane 6: positive control ('Bison type' *Mycobacterium avium* subspecies *paratuberculosis* DNA); lane 7: negative control (deionized distilled water); lane 8: A13 (DNA from a suspected patient stool culture); lane 9: A14 (DNA from a suspected patient stool culture); lane 10: A17 (DNA from a suspected patient stool culture); lane 11: CDF1 (DNA from a healthy person stool culture); lane 12: CDF2 (DNA from a healthy person stool culture).

This study revealed high presence of MAP in North Indian human population with and without Crohn's disease. Similar high prevalence of MAP has been reported in Crohn's disease patients and controls by other workers.[13,17,40,41] Sensitivities of culture and ELISA were similar for the detection of MAP in humans. Sanderson et al.[42] reported that culture was less sensitive as compared to PCR. IS900 PCR has frequently been used to confirm MAP in cases of Crohn's disease and controls. Mishina et al.[43] employed reverse transcriptase PCR using MAP-specific primers (IS900) on total RNA from ileal mucosal specimens of Crohn's disease patients and controls with ulcerative colitis and colon cancer. Bull et al.[13] showed that the detection rates for MAP in Crohn's disease depended critically on the validation of the methods used. High proportions of MAP have been reported by using improved culture and IS900 PCR.[44] When these tests are optimal almost everybody with Crohn's disease could be found to be infected with MAP.[45] The presence of MAP colonization of the gut in a minority proportion of persons without Crohn's disease is consistent with widespread environmental exposure to these pathogens, as exemplified also in the population biology of *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Helicobacter pylori*.[45]

In a significant finding, MAP was isolated from two of three blood clots left after harvesting of serum from Crohn's disease patients. These individual were also positive in biopsy and stool cultures. In a study on MAP bacteremia, Naser et al.[14] cultured MAP from the blood of 14 (50.0%) patients with Crohn's disease and two (22.0%) with ulcerative colitis. Presence of MAP in blood from patients with Crohn's disease should not be surprising since in animals with Johne's disease, MAP infection is systemic.[46] MAP is secreted into milk[47] and infiltrates the extra-intestinal tissues and the bloodstream of animals.[46] Viable MAP has been cultured from commercial pasteurized milk in UK[48] and USA,[49] and from potable chlorinated municipal water in USA.[43] These may be potential sources of MAP, if it is eventually accepted that MAP is zoonotic.[50] MAP has also been cultured from the milk of two women with active Crohn's disease.[51]

As reported by Bernstein et al.,[30] the goat ELISA[27] initially developed for screening of goats for Johne's disease was successfully adapted to screen human beings. Using two antigens of animal origin (goat and bovine), the seroprevalence of MAP was high in human subjects from North India,

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therefore indicating exposure from an animal source. However, indigenous antigen was superior to commercial antigen in detecting MAP in Crohn's disease patients. Uniquely, agreement between the two antigens was high when used in human subjects, but was either low or nil when used in goats[27] and buffaloes.[52] Similar to the present findings, Chiodini et al.[10] reported significantly higher levels of IgG antibodies to MAP lysate in animal attendants than in healthy humans. In animals, culture was the most sensitive of the multiple diagnostic tests used,[53] and positives in ELISA were always lower than in culture.[52,53] Though Hermon-Taylor reported 28 different strains of MAP and some specific to particular species like sheep and cattle,[12] in North India, MAP 'bison type' was the most prevalent genotype in animals.[52,53] Collins et al.[40] detected MAP in significantly higher numbers of patients with Crohn's disease than in controls. Similarly in this study, all the Crohn's disease patients were positive by ELISA as compared to 80.0% by culture. Bernstein et al.[30] in a population-based study, reported 35.0% seropositivity rates for all groups, unlike the present findings and there was no difference in rates between Crohn's disease patients, ulcerative colitis patients, and healthy controls or non-affected siblings. Though BCG vaccination status for randomly selected individuals was not known, Collins et al.[40] showed that BCG vaccination did not affect the level of serum antibody to MAP. BCG vaccination is, however, not popular in India. High presence of MAP was recorded in culture and all the isolates were characterized as MAP using IS900 PCR. Similarly Collins and co-workers,[40] in a subset of IBD patients, reported association with MAP or similar fastidious mycobacterial species. A reported case control colonoscopy plus biospy study of a nested cohort of the study population with a nested PCR for MAP revealed a positive rate of 32.0% among controls,[54] a rate that is comparable to the seropositive rate in another study.[30] A PCR study from the UK reported a positive rate of 26.0% in control tissues.[13]

Presence of MAP in pasteurized milk supplies may be an important link to the high presence in humans.[48,55,56] Widespread infection with MAP in domestic animals has been reported in the Agra region of North India.[18,19,23–27,52,53] An additional potential source for humans to contract MAP infection is from drinking water.[43] High (21.0–70.0%) herd prevalence of MAP has been reported from Western Europe and Northern America.[12] Therefore, either in the food chain or in water supplies, it is inevitable that humans sharing the same geographic areas with animals that are extensively infected, are regularly exposed to MAP. This first Indian study is in agreement to some extent with the observations of Hermon-Taylor and co-workers[44] that ''problems caused by MAP constitute a public health issue''.

Conclusions

IS900 characterization of positive cultures in stool and biopsies from confirmed cases of Crohn's disease conclusively prove the association between MAP and Crohn's disease. The positive cultures from animal attendants with suspected infection and healthy persons confirm the high presence of MAP in the investigated human population of North India. This is the first report of ZN positive forms of MAP in animal attendants. MAP was detected in all the Crohn's disease patients by ELISA using indigenous PPA. The high reactivity of commercial PPA indicated involvement of a different genotype in humans. Indigenous PPA from MAP 'bison type' genotype of goat origin can be useful in screening for Crohn's disease. This study is the first to report on association between MAP and Crohn's disease in India. High presence of MAP in animal attendants and healthy persons shows that besides association with Crohn's disease, MAP may also play a role in cases of colitis and other abdominal ailments in India.

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