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SHORT COMMUNICATION

Therapeutic management of *Mycobacterium avium* subspecies *paratuberculosis* infection with complete resolution of symptoms and disease in a patient with advanced inflammatory bowel syndrome

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Abstract

Background A 26-year-old male had a history of frequent bowel movements, mushy stool with mucus and loss of 25 kg body weight in 6 months was diagnosed as a case of inflammatory bowel disease (IBD). The patient did not respond to routine and standard treatment for IBD. His condition was steadily deteriorating, and he was in a very precarious state when he reported to us.

Methods Upon laboratory investigation by using IS900 specific PCR [which is specific for *Mycobacterium avium* subspecies *paratuberculosis* (MAP)], the blood and stool samples were found negative. However, the presence of low titer MAP-antibodies by indigenous ELISA were found followed by detection of the typical acid-fast MAP bacilli (with 3 + or 4 + grade) microscopically. The MAP stool culture was positive after 6 months incubation. The biotyping by IS*1311* specific polymerase chain reaction restriction enzyme (PCR-RE) confirmed infection with 'Indian Bison Type Genotype', a dominant biotype infecting the domestic livestock population of India. Standard anti-MAP therapy was initiated under supervision of the treating physician. The drug of choice in prescribed treatment regimen included Isoniazid (5 mg/kg), Rifampicin (10 mg/kg), Ethambutol (15–25 mg/kg) once a day for 24 weeks and Clarithromycin (250 mg)/Levofloxacin (250 mg) twice a day for 6 weeks.

Results Following treatment, the patient started improving progressively with reduction in bowel movement frequency and gained body weight with an enhanced appetite propensity. Upon follow-up of the patient after 1 year of treatment, stool-microscopy and stool-culture were found negative for MAP. Till the recent past, the patient was further monitored for disease relapse, if any.

Conclusions This patient has experienced a complete resolution of IBD using a combination of anti-MAP antibiotics. The initial detection of heavy shedding of acid-fast MAP bacilli and typical colony morphology with its characterization obtained from culturing of stool sample indicated the infection of MAP. Interestingly, the present case is one more example of the linkage of demonstrable MAP infection treated with anti-MAP therapy in the presence and then absence of disease in the human host.

Keywords *Mycobacterium avium paratuberculosis* (MAP) · Crohn's disease · Antibiotic therapy · Stool culture · Microscopy · ELISA · IS*900* PCR and IS*1311* PCR-RE

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Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is one of the leading pathogens causes Crohn's disease (CD), a chronic inflammatory disease of the gastrointestinal tract. MAP primarily infects domestic ruminant and camelids animals and is considered to cause the Johne's disease (JD) in these animals, however it also has been reported in the other animals, including primates and humans [1–4]. MAP is endemic in the domestic livestock population of India [3]. JD is a major cause of low per animal productivity [5] and a serious threat to the human population [3, 6, 7]. MAP severely infect in individuals suffering from co-morbidities and autoimmune disorders like IBD, Crohn's disease (CD), Ulcerative colitis (UC), Type 1 diabetes mellitus (T1DM), Thyroiditis, and Rheumatoid arthritis [3, 8–10]. MAP potentially infect susceptible humans resulting to triggering of detrimental inflammatory responses in human gut by exposure to kill MAP bacilli and the potentially trigger detrimental inflammatory responses in other organs. Reservation about the existence of CD is being strongly refuted by recent reports from different regions of the India [11]. In a retrospective community-based study on the Indian migrant population who traveled in different countries, the minimum incidence of the CD was reported as $0.14/10^5$ persons per year Probert et al. [12]. Depending on the region, the reported annual incidence of CD ranged from 1 to 10 cases per 100,000 populations and apparently alarmingly increasing [13]. In a cross-sectional study Kappelman and his group reported prevalence rate of CD in children (<20 years of age) was 58 and 241 in the adult (aged 20 years and older) population of USA [14]. However, information is deficient for the incidence rate and CD patient's treatment outcome in India.

Different antibiotic combinations have been tried against the intracellular atypical mycobacteria. The multidrug therapy (MDT) that included anti-tuberculosis antibiotics used for the treatment of CD has been found ineffective [15, 16]. With more specific anti-MAP triple therapy combining of drugs, Rifabutin, Clarithromycin and Clofazimine have generated a favorable response in CD patients [17, 18]. Other studies reported 28% remission and 31% response to the treatment of CD by using a Rifabutin and Macrolide Antibiotic Therapy (RMAT) regimen accompanied with probiotics [19]. Singh et al. [20], reported the concurrent resolution of the disease and MAP infection in patients undergoing with multi-antibiotic therapy as a regime to treat tuberculosis and CD. Kuenstner et al. described two patients with disease, one with CD and the other with complex regional pain syndrome, and MAP that was cultured from their blood. Following anti-MAP therapy that included a combination of ultraviolet blood irradiation and anti-MAP antibiotics, the diseases resolved, and MAP could no longer be cultured from their blood specimen [21]. This case report evaluated an anti-MAP regimen to treat a patient with MAP infection and advanced IBD or CD.

History

A 26-year-old male patient, native of district Bharatpur (27.22°N 77.48°E), Rajasthan, India, had a history of frequent bowel movements (5–6 times per day) along with passage of loose (mushy) stool with heavy mucous production or only mucus. From September 2014 to August 2015, the patient consulted several gastroenterologists and sought health care in hospitals in Bharatpur and Jaipur (26.9°N 75.8°E), Rajasthan, India for the treatment of his ailment. The patient had a history of animal contact and consumed raw milk for 2 years.

He was referred to us by our first patient from Bharatpur who was successfully treated for MAP infection and CD (report published by [20]). At the initial visit during August 2015, he was suffering from severe depression in addition to his clinical symptoms and signs of CD that included diarrhea, severe weakness, weight loss (25 kg in last 6 months), fatigue and anorexia. The inability to perform day-to-day activities made him to confine in his home/bed. He was extremely exhaustion as he covered 35 km to reach our laboratory in a car. He had severe weakness and was unable to walk independently. His prognosis was grave, and it was doubtful whether he could undertake the rigors of anti-mycobacterial therapy.

Screening of the patient for MAP infection

Prior to treatment

Upon initial intervention with the patient during August 2015, complete clinical history was recorded, blood, serum and stool specimen were collected to assess to find the presence of MAP using laboratory diagnostic assays viz., Stool microscopy, Stool culture, IS*900* PCR (DNA extracted from stool and blood) and indigenous serum ELISA (i ELISA) Kit (as developed by Singh et al. [22]). There are four elementary tests (microscopy, culture, ELISA and PCR since 1984, culture since 1988, ELISA since 1992 and PCR since 2000) being used routinely depending on the sample type and requirement. Clinical samples were processed for respective investigation at the microbiology laboratory of the Central Institute for Research on Goats (CIRG), Mathura, Uttar Pradesh, India.

Monitoring of the patient during treatment (180 days post treatment) and at the end of treatment (360 days post treatment main course)

During the treatment period and at the completion of 1 year of treatment, the response to anti-MAP regime was assessed by the presence of the clinical symptoms, physical condition of the patient (gain in body weight) and by the presence or absence of bio-load of MAP in blood and stool. The MAP presence/bio-load was monitored by using stool microscopy, culture, and specific PCR (IS*900*) and immunological parameters by the i_ELISA Kit.

IS900 PCR

DNA from human blood sample was isolated and subjected to specific IS900 PCR as per Singh et al. [2]. MAP specific primers used were forward primer-P90 5′-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3′ and 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 MgCl2, 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 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s 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.

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 Johne's disease as per Singh et al. [22]. Serum samples from earlier studies and collected from Crohn's disease patients confirmed for MAP infection in IS900 PCR and healthy MAP negative person were used as positive and negative 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 [23] to determine the status of MAP infection. Serum sample in the S/P ratio range (≥ 0.40) was categorised as cut-off and were considered positive for MAP infection.

Microscopic examination

Two grams of stool sample was grounded in sterilized distilled water (12 mL) in sterilized pestle and mortar. Grounded material was centrifuged at 1557×*g* for 1 h at room temperature; smears prepared from middle layer, stained with Ziehl–Neelsen (ZN) staining as per Parashar et al. [24] and were observed under oil immersion for presence of pink short rods indistinguishable to MAP.

Culture

Middle layer was also decontaminated using 0.9% hexa decyl pyridinium chloride (HPC) After decontamination, the sediment pelleted was inoculated on Herrold egg yolk medium with mycobactin J (HEYM) as per method of Whipple et al. [25] with some modifications and incubated at 37 °C upto 8–16 weeks.

Treatment and management of MAP infection

An initial treatment regimen with anti-MAP therapy was initiated under the supervision of a local physician that included Isoniazid (300 mg), Rifampicin (450 mg), Ethambutol (800 mg) once a day, for 24 weeks and Clarithromycin/Levofloxacin (250 mg) twice a day for up to 6 weeks. The patient was also treated for depression with anti-depressant, anti-anxiety medications. The anti-depressant regimen follows: Chlordiazepoxide (5 mg) thrice a day for 24 weeks. Afterwards, the patient was administered with Chlordiazepoxide (5 mg) thrice a day and Amitriptyline (25 mg) once a day, and Clonazepam for 48 weeks. In addition to these, the patient was also administered with the drug combinations of pantoprazole/rabeprazole and escitalopram (Table 1). After 48 weeks, a maintenance treatment regimen with recommended dosage, Chlordiazepoxide (5 mg), Clidinium bromide (2.5 mg) and Dicyclomine (10 mg) twice a day was initiated.

After confirmation of laboratory diagnosis for active MAP infection, we discussed the treatment strategy with the physician (Dr. Prabhat Agrawal) and prescribed anti-MAP therapy for him. However, the patient's condition was very fragile and he initially experienced continuing and worsening diarrhea. The patient was in Bharatpur, 35 km from Agra (27.18°N 78.02°E), Uttar Pradesh, India (where his treating physician, Dr. Prabhat resides) and also 35 km from Farah town (where the laboratory diagnosis performed in CIRG, Mathura, Uttar Pradesh, India). Early in his therapy duration, the patient contacted his physicians about his precarious condition and his physician admitted him to a nursing home in Bharatpur and then instructed another treating physician there to administer intravenous fluids (without anti-MAP therapy). Within one week, his clinical condition improved. He was slowly restarted on anti-MAP therapy and within 20 days when his condition improved, the full course of anti-MAP therapy was resumed. After receiving written informed consent from the patient, the decision to treat the patient with our approach to after in-house diagnostic assays

diagnosis and treatment was made because of the patient's gave prognosis and his poor response to the standard therapy and with informed consent from the patient. From our own previous experience, we were also confident in our MAP diagnostic methods and anti-MAP therapy [20].

Results

Prior to the initiation of anti-MAP treatment, the patient suffered from IBD/CD and experienced the frequent passage of loose stools with heavy mucus, weight loss and weakness.

Table 1 Summary of treatment regimen up to 48 weeks (12 months)

Drug category	Drug regime	Treatment duration
Antibiotics	Clarithromycin (250 mg)/Levofloxacin (250 mg)	Up to 2 weeks (twice a day) Up to 2–6 weeks (twice a day)
Anti-TB	Isoniazid (300 mg)	Up to 24 weeks (once a day)
	Rifampicin (450 mg)	Up to 24 weeks (once a day)
	Ethambutol (800 mg)	Up to 24 weeks (once a day)
Anti-inflammatory	Mesalamine (1 g) or 5-aminosalicylic acid	4–48 weeks (twice a day)
Anti-depressant and anti-anxiety	Chlordiazepoxide (5 mg)/Etizolam (0.5 mg)/Clonazepam (0.5 mg)	Up to 36 weeks (once a day)
	Amitriptyline (25 mg)	36–48 weeks (once a day)
Anta-acid and anti-ulcer	Pantoprazole or Rabeprazole	7–48 weeks(once a day)

*Along with an above regimen multivitamin, digestive enzymes/probiotics were also prescribed

Table 2 Screening of clinical samples (feces, blood and serum) by multiple diagnostic tests for diagnosis of the presence of MAP at different days post treatment

Tests	0th day	30th day	90th day	360th day
1. ELISA—serum*	Low positive (0.3255)	Suspected (0.2875)	Negative (0.2321)	Negative (0.1721)
a. Status of MAP				
b. OD values (S/P ratio)				
2. Microscopy—stool	3 + to 4 + (heavy)	2 + to 1 + (moderate)	2 + to 1 + (moderate)	Negative
3. Culture—stool**	Multi-bacillary	Not done	Negative[#]	Negative
4. IS*900* PCR-blood***	Negative	Negative	Negative	Negative

*Status of MAP infection as per S/P ratio [23] using 'Indigenous ELISA Kit'

**MAP colonies took 1 year to grow

***Directly from DNA isolated from blood and stool samples

[#]Patient was under anti-MAP treatment

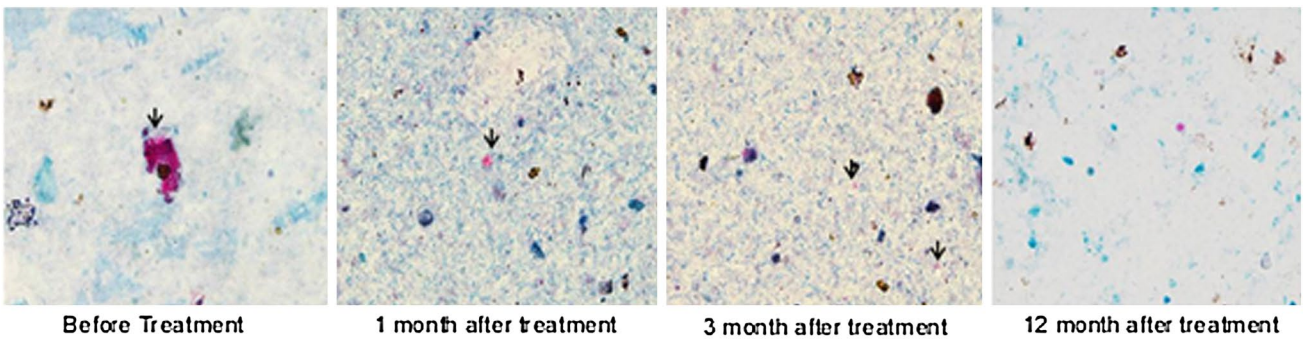

Fig. 1 Microscopic examination of stool (Typical MAP bacilli as seen in ZN staining) **A** Heavy shedding of the typical MAP bacilli before onset of treatment, **B**–**C** Moderate shedding of the typical MAP bacilli after 1-and 3-month treatment, **D** Negative for MAP bacilli at the end of 12 months of treatment

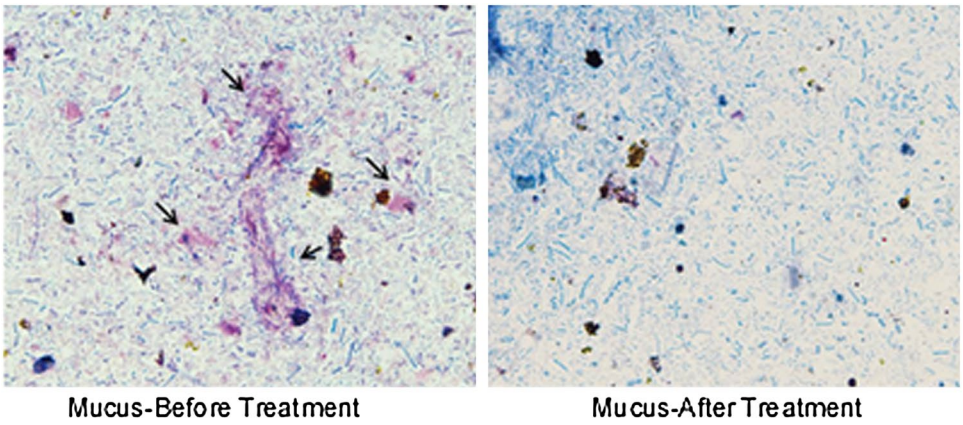

Fig. 2 Microscopic examination of Mucus (shedding of typical MAP bacilli as seen in ZN staining) before and after anti-MAP treatment

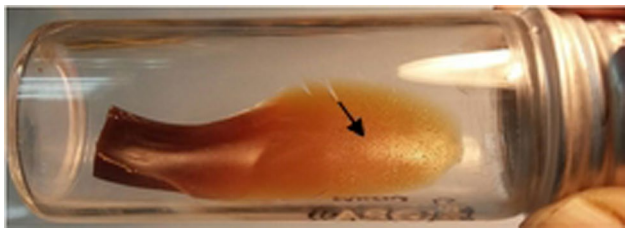

Fig. 3 Typical colonies of MAP visualised at the end of one year of incubation

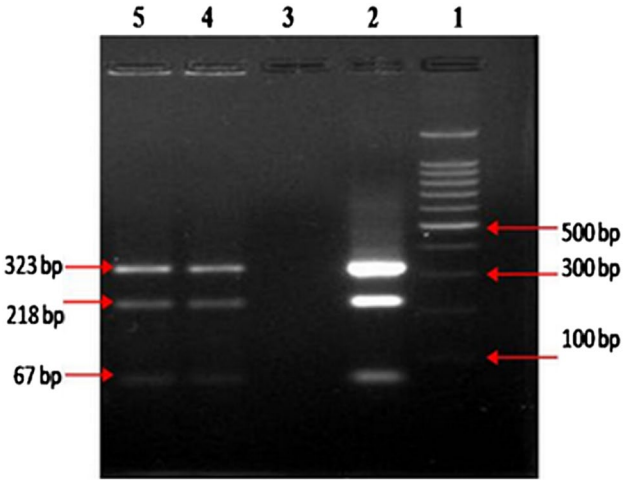

Fig. 4 Biotyping of MAP bacilli (Indian Bison Type) from colonies developed at the end of 12 months on HEY Medium by IS 1311 PCR-RE assay. Lane 1: Molecular Marker (100bp); Lane 2: Positive control (MAP DNA); Lane 3: Negative control; Lane 4 and 5: Test DNA sample

The initial stool sample from August 2015, exhibited heavy shedding (3 + to 4 +) of acid-fast bacilli indistinguishable from MAP, IS*900* PCR tests of blood and stool were negative and the serum sample was low positive for MAP infection by the i_ELISA, as per [23] (Table 2, Figs. 1 and 2). Therefore, based on clinical symptoms, treatment history, stool microscopy findings and 'low positive' result by the i_ELISA, the patient was diagnosed as 'active carrier of MAP infection' causing IBD. However, the first stool sample of the patient obtained before initiation of the anti-MAP treatment was positive by culture (colonies began appearing after 8 months of incubation on HEY media) (Fig. 3). After seeing enough growth of typical MAP colonies grew on HEY media inoculated with stool specimen after 12 months incubation, the isolate was confirmed as MAP by using IS*900* PCR assay. The IS*1311* PCR-RE digestion analysis revealed the resemblance with the "Indian Bison Type" (a dominant biotype strain of India) (Fig. 4).

After 14 weeks of anti-MAP treatment, the patient exhibited a reduction in frequency of stool passages (2–3 times a day), a reduction in stool mucus with an improved appetite propensity (Tables 1, 2 and 3). After 3 months, stool microscopy for MAP was still positive (1 + to 2 +) but showed a reduction in the load of MAP shedding as compared with the sample tested before the onset of anti-MAP treatment however the patient was still sero-negative (i_ELISA). After completion of 1 year of treatment, the patient exhibited progressive improvement by showing a further reduction in stool frequency (1–2 times a day) and a total gain in body weight of 9 kg (Table 3). At this point, follow-up stool microscopy was negative for MAP infection. Interestingly, i_ELISA was negative for MAP and he experienced a complete recovery from clinical symptoms.

Discussion

Our findings describe a case of resolution of IBD/CD following the demonstration and treatment of MAP infection in a human host by using stool culture and microscopy as a diagnostic and treatment monitoring tool in conjunction with serologic tests. Before anti-MAP treatment initiation,

Table 3 Improvement in body weights and stool frequency at different time points after treatment

S. No.	Days (post treatment)	Observation	
		Body weights (in kg)	Stool frequency
1	0th day	Lost up to 25 kg in 6 months (55.0)	5–6 times a day, mucus present
2	30th day (1 months)	Gain up to 1 kg (56.0)	2–3 times a day, mucus present
3	90th day (3 months)	No gain (55.0)	2–3 times a day, no mucus
4	180th day (6 months)	Gain up to 3 kg (57.0)	1–2 times a day, no mucus
5	360th day (12 months)	Gain up to 9 kg (64.0)	1–2 times a day, no mucus
6	540th day (18 months)	Gain up to 10.6 kg (68.6)	1–2 times a day, no mucus

the clinical condition of the patient steadily declined while on standard treatment for IBD. The stool specimen was initially negative by culture, but prolonged incubation for more than 8 months could result culture positive for MAP. Blood and stool specimens were negative for MAP by IS*900* specific PCR. However, titer value was low in range (S/P ratio < 0.21) by i_ELISA but routine microscopy for stool was positive. Therefore, on the basis of clinical symptoms, treatment history and stool microscopy [wherein he was excreting large quantities of typical acid-fast short rods (cocco-bacilli) indistinguishable from MAP], the patient was diagnosed as an active case of MAP infection. MAP isolates are very slow growing as compared to other Mycobacterial strains. Initially, stool culture on HEY media was negative at incubation for 1 month; minute, typical, MAP colonies were seen when culture media were further incubated for 6 months, however sufficient colonies were seen after 1 year of incubation. The colonies were harvested to confirm MAP by performing the biotyping, PCR-REA and PCR assays.

The 'low positive' titer by 'i_ELISA' for MAP infection might be because of the damage or suppression of the immune system resulted from prolonged and heavy MAP infection or treatment with steroids. Upon following 3 months anti-MAP treatment, the condition of the patient started improving. A similar condition of anergy has been reported in animals suffering with advanced stages of JD [23]. Therefore, a complete assessment of clinical state and its correlation with multiple laboratory-based investigations are essential in deciding and confirming the MAP diagnosis, subclinical condition of MAP infection in the animals and human may exhibit the dormant stage for the bacilli [10]. The strain similarity and patient response against the specific anti-MAP therapy provides further backup to our diagnostic approach in confirming the MAP infection, leading to IBD/CD symptoms and disease. Recently, Singh et al. [20], described a patient with a history of frequent bowel movements who was negative by IS*900* PCR and 'i_ELISA', yet responded to the treatment with standard anti-MAP drugs. Similarly, Singh et al. [26], described a case report wherein a cow with continuous diarrhea for 3 years and a calf exhibiting stunted growth were negative by microscopy and 'i ELISA' yet showed prophylaxis against MAP vaccine. The indigenous vaccine developed using the 'Indian Bison Type' MAP biotype of goat origin is both therapeutic and preventive management in domestic livestock (cows, goats, buffaloes, and sheep) animals [26, 27]. The combination of biotyping, molecular testing (as performed in this case), stool microscopy and the i ELISA are an ideal option as laboratory tests for the MAP diagnosing. Both the tests (stool microscopy and i_ELISA) have already been used to study the efficacy of our vaccine against MAP in domestic livestock animals [26].

Farmers and rural population in India maintain close contact with the domestic livestock animals, it is because of milking practices in cattle, buffalo, goat and share the household space with animals because of limited socio-economic conditions. Studies of the bio-burden and biotyping of MAP in large populations of domestic livestock, wild ruminants and other animals in the past 32 years have shown that JD is widely prevalent and endemic in the domestic livestock population of the India [3]. Additionally, mass screening of the human population in this part of India has revealed a high level (> 30.0%) of MAP infection by 'i ELISA' [3]. Laboratory screening of the milk and its product also revealed a high MAP bio incidence [28, 29]. Biotyping of MAP strains in the past 15 years, in livestock animal species, including wild, ruminants, domestic, human being, milk and milk products and geographical regions consistently represented the "Indian Bison Type" strain as the most prevalent MAP biotype of India [3, 29, 30]. In paucity of the surveillance, monitoring and control program for the JD, the circulation of MAP strain has sharply increased, especially in the domestic livestock [3]. The following factors such as poor hygiene and sanitary conditions, lack of potable water, overpopulation of domestic livestock at 567.27 million (highest and 14.6% of world population), overpopulation of human (second highest and 17.5% of world human population) on 2.8% land and 4.0% water resources that too in absence of a paratuberculosis control program is hypothesized for increase in this number.

Studies on MAP biotype revealed the presence of most pathogenic SSR repeats (7G4GGT) [31]. This 'Indian Bison Type' biotype belongs to SSR profile, wherein 7G4GGT repeats associated with highly pathogenic strains of MAP

circulating worldwide. Large scale prevalence and high bio-load of MAP in domestic and wild ruminants, in their milk and products and also in the human population and environment, results in a wider-host range with high pathogenicity and endemicity of MAP infection in the Indian "eco-bio-system". Periodical screening of the samples obtained from animals, food, environment, human beings supported by published case reports depict the supplementary evidence of a pathogenic role of MAP in the humans [10]. Our findings on native biotype 'Indian Bison Type' of MAP in the last 32 years revealed not only a broad host range but also wider prevalence rate as a multi-species pathogen [3] and also as important "food-borne pathogen" [29].

In the absence of MAP control and surveillance programs, the bio-load of MAP is increasing at a rapid pace, not only in animals, but also in food items including milk and milk products (ice-cream, milk powder, flavored milk, cheese, buttermilk), in environment (river water and soil) and human population [6, 29]. In order to control the spread of infection in human population, it is very essential to control the infection in animals because MAP is transmitted via both the vertically and horizontally routes. It will also be necessary to revise standard pasteurization conditions since the current standards are not enough to kill MAP.

Recent studies demonstrated the presence of MAP in healthy individuals as well as patients with various illnesses including CD [3, 32]. These findings show humans, and other species are susceptible to infection with MAP. Cumulative studies suggest that MAP could play a role in pathogenesis directly as the etiologic agent and indirectly through mimicry where secreted products contribute to the development of autoimmune and other disorders [8], 9]. Until now, our focus has been in establishing a correlation between the prevalence of MAP in livestock and the occurrence of CD and other diseases [3, 7, 30]. Despite of the controversy, whether MAP is a zoonotic pathogen and the causative agent for CD, no program has, thus so far been initiated to explore the potential of different anti-mycobacterial therapies to cure patients with CD. In India, steroids are the most common therapy for induction (in 37%) followed by 5-aminosalicylates (ASA) (17%) [33]. Interestingly, 14% receive anti-tuberculosis treatment (ATT in the initial phase and 38% patients who were initially treated with ATT had some symptomatic response but most of these patients subsequently relapse. In India, majority of patients receive 5-ASA as maintenance therapy either alone (63%) or in combination with azathioprine (AZA) (21%) with a majority maintaining remission. The reason for the response to 5-ASA is unclear but may be due to anti-MAP activity described by Robert Greenstein and Sheldon Brown [11, 34]. There are isolated reports of the use of biological from India [35]. However, in India, their role both in induction and maintenance have not been studied in properly designed trials. Finally, massive controlled clinical trials using the diagnostic tool and treatment regimen of this case report are required to better assess the role of MAP in human disease. The successful treatment and recovery of this patient and others has given us confidence in our diagnosis, therapy, and management of patients with IBD.

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Author contributions SG carried out all the experiments, prepared figures and drafted the manuscript. KKC collected the data and analysis. PA senior physician designed the drug regiment/therapeutics, JTK and DP participated in data analysis and interpretation of results. SG, DP and SVG design the study, participated in data analysis, and drafted the manuscript.

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Declarations

Conflict of interest The authors declare there were no financial or commercial conflict of interest associated with the report of the present study.

Informed consent Written informed consent was obtained from the patient for treatment and for publication of this report and accompanying images.

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