

# Absence of *Mycobacterium avium* ss *paratuberculosis*-specific IS900 sequence in intestinal biopsy tissues of Indian patients with Crohn's disease

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## Abstract

**Background and Objective** The role of *Mycobacterium avium* ss *paratuberculosis* (MAP) in the etiopathology of Crohn's disease (CD) remains controversial, because of conflicting reports demonstrating the presence of MAP-specific insertion sequence from intestinal biopsy tissues of patients clinically diagnosed for the disease. The present study was carried out to investigate the presence of MAP DNA in the intestinal tissues of CD patients to ascertain the relevance of MAP in Indian patients with CD.

**Methods** Patients diagnosed as CD at our institute were recruited. Healthy individuals without inflammatory bowel disease served as controls. Mucosal biopsy specimens were collected from ileum and colon in duplicates and subjected to histopathological examination and polymerase chain reaction (PCR) amplification. Total DNA (81 CD patients, 85 healthy individuals) and total RNA (12 CD patients, 12 healthy individuals) isolated from tissue specimens was used for amplification of MAP-specific IS900 by nested PCR.

**Results** MAP-specific IS900 DNA and RNA could not be detected by nested PCR in the intestinal tissues of any patient with CD.

**Conclusion** Our results do not support the etiological role of MAP in the pathogenesis of CD in Indian patients.

**Keywords** Inflammatory bowel disease · MAP-specific IS900 · *Mycobacterium avium* ss *paratuberculosis*

## Introduction

The prevalence of Crohn's disease (CD), when compared with ulcerative colitis, is on the rise in various populations. Even though the etiology of CD remains unclear, previous studies indicate that mycobacterial infection and/or chronic activation of immune system may contribute to the disease. Detection of mycobacterial species in intestinal biopsy tissues from CD patients has earlier revealed the presence of various mycobacteria including *Mycobacterium avium* subspecies *paratuberculosis*, *Mycobacterium avium*-intracellulare, *Mycobacterium chelonii*, *Mycobacterium fortuitum* and *Mycobacterium kansasii*.<sup>1</sup> Of the above, *Mycobacterium avium* ss *paratuberculosis* (MAP) is a causative agent for chronic granulomatous inflammation of the intestines (Johne's disease) in animals including primates.<sup>2–5</sup> Clinical similarities between CD of humans and Johne's disease of animals indicate that MAP could be a causative agent of CD. Hence, it is crucial to establish whether MAP is involved in the etiology of CD.

Identification of MAP involves either culturing the organism, or detecting MAP-specific antibodies or MAP-specific DNA sequences. As MAP is a slow-growing organism, many investigators have relied on the use of MAP-specific IS900, an insertion sequence that is characteristically present in multiple copies and considered very specific to *M. avium* ss *paratuberculosis*.<sup>6–8</sup> Employing this approach, several investigators reported that 13% to 100% of CD patients tested positive for MAP.<sup>9–22</sup> In contrast, several others have failed to demonstrate MAP DNA in tissues obtained from CD patients.<sup>23–29</sup> In view of the controversial relevance of MAP in the etiology of CD the present study was conducted.

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## Methods

### Patients

Eighty-one patients of Indian origin reporting to our institute, which is a tertiary care referral center, between July 2007 and June 2008 were diagnosed as CD based on clinical, histopathological, and colonoscopic features.<sup>30</sup> Eighty-five healthy individuals undergoing screening ileocolonoscopy for colorectal cancer formed the control group. Informed consent was obtained from all the subjects. The protocol was approved by our Institutional Review Board and Institutional Ethics Committee. Comparative clinical features of the control and CD groups are presented in Table 1. At the time of colonoscopy, two bits of mucosal biopsy were taken from each site of lesion in the ileum, ascending and descending colon. One biopsy piece was collected in formalin and sent for histopathological examination, while the other was collected in saline and stored at  $-80^{\circ}\text{C}$ . Mucosal biopsies from ileum and colon were also collected in RNeasy (Qiagen, GmbH, Hilden, Germany) and stored at  $-80^{\circ}\text{C}$  in an additional set of 12 CD patients and 12 healthy individuals for RNA analysis.

### Materials

The total RNA isolation Nucleospin RNA II kit was from Macherey-Nagel, Duren, Germany. Two sets of primers (Table 2) were procured from Operon Biotechnologies, Cologne, Germany. MAP DNA, isolated from culture extracts of *Mycobacterium avium* ss *paratuberculosis* (ATCC 43015), was used as positive control DNA (kind gift from CDFD). All other chemicals were of molecular

**Table 1** Demographic and clinical features of participants

|                                   | Crohn's disease | Control subjects |
|-----------------------------------|-----------------|------------------|
| <b>Demographic details</b>        |                 |                  |
| Number of participants            | 81              | 85               |
| Age (years) (means [SD])          | 55 (10)         | 59 (11)          |
| Male:female                       | 53:28           | 62:23            |
| <b>Clinical presentation</b>      |                 |                  |
| Abdominal pain                    | 74 (91%)        | Nil              |
| Weight loss                       | 56 (69%)        | Nil              |
| Diarrhea                          | 69 (85%)        | Nil              |
| Bleed per rectum                  | 21 (26%)        | Nil              |
| <b>Colonoscopic findings</b>      |                 |                  |
| Ulceration                        | 62 (77%)        | Nil              |
| Stricture                         | 18 (22%)        | Nil              |
| Fistula                           | 11 (14%)        | Nil              |
| <b>Histopathological features</b> |                 |                  |
| Ulceration of epithelium          | 68 (84%)        | Nil              |
| Chronic inflammation              | 71 (88%)        | Nil              |
| Granulomas                        | 22 (27%)        | Nil              |

**Table 2** Primers used for PCR amplification of MAP DNA

|                                                   |  |
|---------------------------------------------------|--|
| First PCR (to generate 398 bp fragment)           |  |
| Forward primer: 5' – GTTCGGGGCCGTCGCTTAGG 3'      |  |
| Reverse primer: 5' – GAGGTCGATCGCCCACGTGA 3'      |  |
| Second PCR (to generate 298 bp internal fragment) |  |
| Forward primer: 5' – ATGTGGTTGCTGTGTTGGATGG 3'    |  |
| Reverse primer: 5' – CCGCCGCAATCAACTCCAG 3'       |  |

biology grade obtained from Sigma Chemicals, St. Louis, MO, USA. RNA later was purchased from Qiagen.

### Isolation of DNA from tissues and bacterial cultures

Mucosal biopsy bits (~20 µg) were decontaminated with hexadecyl pyridinium chloride, homogenized in lysis buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme) and incubated for 30 minutes at  $37^{\circ}\text{C}$ . Proteinase K was added to the lysate, followed by incubation at  $56^{\circ}\text{C}$  for 30 minutes and further incubation for 15 minutes at  $95^{\circ}\text{C}$ . To the above lysate, 200 µL of 96–100% ethanol was added, followed by loading the contents to spin columns (QIAamp; Qiagen). DNA was then eluted from the columns as per manufacturer's instructions. DNA was also isolated from *M. paratuberculosis* ss *avium* positive culture obtained from ATCC 43015 using the above protocol.

### Total RNA isolation

Total RNA was extracted from mucosal biopsies using Nucleospin kits as per the support protocol for total RNA preparations from bacterial cells. First strand cDNA (first strand DNA synthesizer kit, Fermentas, Germany) was synthesized at  $37^{\circ}\text{C}$  for 60 minutes by reverse transcription using total RNA as the template and reverse primer of 398-bp fragment.

### IS900 amplification

Polymerase chain reaction (PCR) was used to generate IS900 MAP-specific 398-bp fragment, using total DNA as the template as per the conditions described below. The reaction mixture consisted of a total volume of 20 µL PCR buffer (100 mM Tris-HCl, pH 8.9), 25 mM  $\text{MgCl}_2$ , 500 mM KCl, 20 pmol of primers, 1 unit of Taq DNA polymerase and 200 ng of isolated DNA as the template. Thirty-five amplification cycles were performed in thermal cycler (MJ Research, Waltham, MA, USA). Each cycle constituted of denaturation at  $94^{\circ}\text{C}$  for 60 seconds, annealing at  $61^{\circ}\text{C}$  for 90 seconds and an extension of  $72^{\circ}\text{C}$  for 90 seconds. To increase the sensitivity and specificity further, the first PCR product was subjected to nested PCR using specific primers (Table 2) to amplify IS900-specific internal 298-bp fragment.<sup>31</sup> Sterile TE buffer was used as template for negative

control to eliminate laboratory contamination in all the PCR reactions. The PCR method was validated by including MAP DNA to samples obtained from both healthy and CD patients which were otherwise negative for amplification of IS900. Similarly, cDNA reverse transcribed from the total RNA was subjected to IS900 amplification as above.

## Results

Crohn's disease was diagnosed in 81 patients (mean age 55 [SD 10] years; 53 men) based on standard clinical, histopathological, and colonoscopic criteria (Table 1). Mean duration of symptoms was 8 (SD 2) months. CD patients presented with one or more symptoms which included abdominal pain in 91% and severe weight loss in 69%. On colonoscopic examination, inflammation, ulcerations, and strictures were observed either in colon (80%) or in ileum (20%). Histopathological examination of biopsy specimens revealed ulceration of epithelium, non-caseating granulomas, crypt-related inflammation and architectural distortion (Fig. 1) in CD patients. No such aberrations could be detected in control group, either by colonoscopic

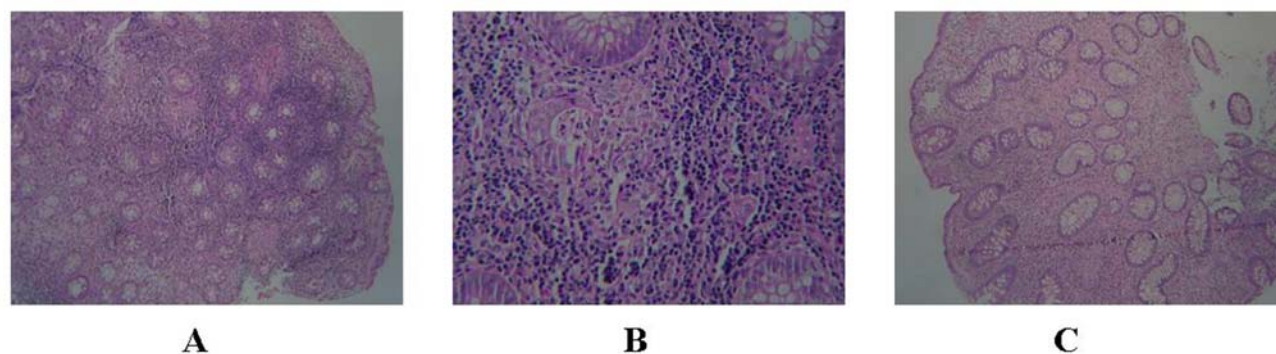
examination or upon histopathological examination of mucosal biopsies (Table 1).

Clinical, colonoscopic, and histopathological findings in the CD patients enrolled for RNA analysis were similar to patients recruited for DNA analysis. None of the patients were on antitubercular or anti-inflammatory medications.

The 298-bp sequence, unique for IS900 of *Mycobacterium avium ss paratuberculosis*, could not be detected upon amplification using DNA samples obtained from either control or CD patients (Fig. 2). To ensure that such a finding did not arise from false negatives, PCR amplification was repeated after including MAP DNA in PCR reaction mixtures. Amplification of 298-bp sequence in the DNA samples to which MAP DNA was added indicates absence of PCR inhibitors (Fig. 3). In corroboration with the above result, RT-PCR amplification of the total RNA also did not amplify IS900 sequence (Fig. 4).

## Discussion

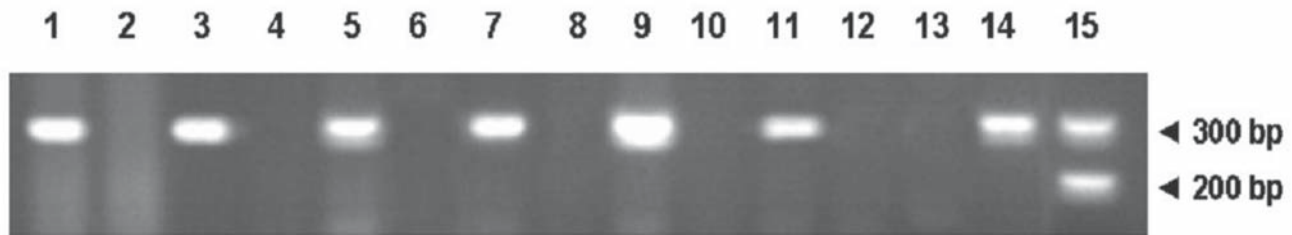
Our results suggest that infection of gastrointestinal tract with *Mycobacterium avium ss paratuberculosis* may not be



**Fig. 1** (A) Mucosal biopsy from patient with Crohn's disease shows mucosal irregularity, crypt activity, crypt destruction and mucosal granulomas (H&E, 40 $\times$ ). (B) High power showing crypt abscess and crypt destruction with diffuse lymphoplasmic cells in lamina propria (H&E, 400 $\times$ ). (C) Crypt architectural distortion and mucosal granuloma seen in the biopsy (H&E, 40 $\times$ )



**Fig. 2** Nested PCR for detection of MAP DNA in intestinal tissues. IS900-specific PCR amplification of DNA from mucosal biopsies of CD patients and healthy individuals. Lane 1–9: Crohn's disease tissues, Lane 10–13: Healthy individual tissues, Lane 14: Negative control, Lane 15: Positive control, Lane 16: 100-bp Ladder



**Fig. 3** Validation of PCR with positive control. PCR was validated by adding positive DNA to the negative samples. Lane 2, 4, 6, 8, 10, 12: Negative samples, Lane 1, 3, 5, 7, 9, 11: Negative samples + Positive control, Lane 13: Negative control, Lane 14: Positive control, Lane 15: 100-bp Ladder



**Fig. 4** RT-PCR for detection of MAP RNA in intestinal tissues. IS900-specific PCR amplification of cDNA reverse transcribed from RNA of mucosal biopsies of CD patients and healthy individuals. Lane 1–9: Crohn's disease tissues, Lane 10–13: Healthy individual tissues, Lane 14: Negative control, Lane 15: Positive control, Lane 16: 100-bp Ladder

the etiological factor in CD. MAP-specific IS900 sequence could not be detected in intestinal biopsy specimen collected from patients diagnosed for CD as well as in the control group. Even though the clinical profile of CD patients was in conformity with the histopathological and colonoscopic criteria, absence of MAP-specific DNA/RNA upon PCR amplification suggested that the etiology of CD cannot be ascribed to infection with MAP. These results are in agreement with earlier reports of Ellingson *et al.*<sup>32</sup> who examined intestinal tissues from CD of humans and Johne's disease of cattle by histology, immunohistochemistry, and PCR analysis using the MAP-specific IS900 sequence. Contrarily, the presence of MAP DNA was documented in Indian populations, including healthy adults, animal attendants as well as in patients suspected for CD.<sup>33</sup> However, the limited number of CD patients in the study ( $n=5$ ), use of colony PCR but not nested PCR, and the identification of MAP-specific IS900 in all tissue samples examined, including 80% of CD patients and 23% of control subjects, limits a conclusion relating MAP as the causative agent for CD in humans. The fact that MAP DNA was detected even in healthy individuals in some regions indicates its co-existence with other microflora.<sup>34</sup>

The present finding, indicating that MAP is not the causative agent for CD in humans, finds additional credence

from the recent report by Selby *et al.*<sup>35</sup> Considering that long-term remission of clinical manifestations should occur upon clearance of the infection with combination antibiotic therapy (clarithromycin, rifabutin, and clofazimine), this group conducted a large scale, placebo-controlled, double-blind, randomized trial with a large population of CD patients over a 2-year period; the long-term relapse rates were similar in the antibiotic and placebo arms. The authors thus concluded that their study did not support any role for MAP in CD. This indicates that the etiology of CD in humans is more complex. It is also likely that MAP transiently exists in the intestinal tract of normal individuals.<sup>34</sup>

We did not detect a single positive PCR product for MAP-specific IS900, although we employed IS900-specific nested PCR which is most widely used for the detection of MAP DNA in CD patients. One limitation of the study may be the sensitivity of the method. However it has been reported that IS900-nested PCR is able to detect one to two CFU in milk samples.<sup>36</sup> Therefore, the method that we used seems to be adequately sensitive to detect MAP in biopsy specimens. Presence of the 298-bp band in the positive control indicates that the PCR conditions used for amplification were appropriate. The presence of inhibitors is also ruled out as the positive control when added to the MAP DNA-negative samples have amplified and showed

a 298-bp band. Our results are in agreement with recent reports which have shown that MAP is indeed absent in cultures from blood of CD patients.<sup>37</sup>

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