In Situ PCR for *Mycobacterium tuberculosis* in Endoscopic Mucosal Biopsy Specimens of Intestinal Tuberculosis and Crohn Disease

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Abstract

Tuberculosis and Crohn disease are granulomatous disorders affecting the intestinal tract with similar clinical manifestations and pathologic features. We evaluated the use of in situ polymerase chain reaction (PCR) using Mycobacterium tuberculosis *complex–specific primers for IS*6110 *to differentiate these 2 disorders in archival mucosal biopsy specimens. In situ PCR was positive in 6 of 20 tuberculosis biopsy specimens and 1 of 20 Crohn disease biopsy specimens. Staining was localized to a site of granulomatous inflammation in 3 of the tuberculosis specimens and in the Crohn disease specimen. In the other tuberculosis biopsy specimens, positive staining was localized to inflammatory granulation tissue and to a focus of intact mucosa without granulomatous inflammation. The presence of* M tuberculosis *DNA in Crohn disease could be due to coexisting latent tuberculosis or indicate a role for these bacteria in triggering an abnormal immune response. Therefore, in situ PCR is potentially useful to differentiate intestinal tuberculosis from Crohn disease, if the sensitivity is improved.*

Tuberculosis (TB) and Crohn disease (CD) are granulomatous disorders of the intestinal tract that are often difficult to differentiate. Granulomas due to TB are classically associated with caseating necrosis and acid-fast bacilli, but these features are seen in only a subset of cases of intestinal TB.[1] Studies from our laboratory have shown that the size, number, and location of granulomas may be additional features useful in distinguishing intestinal TB from CD,[2,3] but even these are not seen in all cases. Bacterial cultures, immunohistochemical analysis, in situ hybridization, and polymerase chain reaction (PCR) are different techniques used to detect mycobacteria in tissue and improve the specificity of the diagnosis of TB. PCR has the advantage of being faster than bacterial cultures and more sensitive than immunohistochemical analysis and in situ hybridization,[4] but conventional PCR requires nucleic acid extraction and tissue destruction, making correlation with histologic features impossible.[5] In situ PCR, however, enables amplification of target sequences within intact cells and combines high sensitivity with the ability to localize specific DNA in tissues, although its sensitivity may be less than that of conventional PCR.[6] Earlier work from our laboratory used in situ PCR to demonstrate *Mycobacterium tuberculosis* in latent lung infection.[7]

CD is a chronic inflammatory disorder of multifactorial etiology. Infectious agents are one of the postulated causes, and measles virus particles, *Mycobacterium avium* subsp *paratuberculosis, Yersinia pseudotuberculosis,* and *Yersinia enterocolitica* have been shown to be present in intestinal samples of CD.[8-10] *M tuberculosis* was found in 5% of patients with CD in 1 study, yet not found in any patients in another 2.[11-13] The aim of the present study was to test the usefulness of in situ PCR and tissue localization of positive

staining in differentiating intestinal TB from CD on archived endoscopic mucosal biopsy specimens.

Materials and Methods

Study Samples

We selected 20 cases of TB and 20 cases of CD diagnosed on the basis of a combination of radiologic, endoscopic, histologic, and clinical guidelines, including response to treatment, as used in previous studies,[2,3] from the files of the Department of Gastrointestinal Sciences, Christian Medical College, Vellore, India. The clinical records were reviewed at the time of selection of cases by an experienced gastroenterologist (S.P.). The mucosal biopsy specimens studied in each case consisted of five to seven 1-mm-sized fragments that had been fixed in buffered formalin for 12 hours and then paraffin embedded. They were coded for blinding the subsequent PCR analysis. Paraffin-embedded blocks had been stored for 3 to 8 years before the study.

In Situ PCR

Sections, 5-μm-thick, were mounted on silane-coated slides, deparaffinized for 18 hours at 60°C, and sequentially immersed in xylene (30 minutes at 37°C), absolute ethanol, 75% ethanol, 50% ethanol, 25% ethanol, and water. Cells were made permeable by incubation at room temperature in 0.02 mol/L of hydrochloric acid for 10 minutes, followed by 0.01% Triton X-100 for 90 seconds. Proteins were depleted by incubation with 1 mg/L Proteinase K (Gibco, Paisley, Scotland) for 30 minutes at 37°C. The Proteinase was then inactivated by boiling in a microwave for 15 seconds, and sections were plunged immediately into 20% acetic acid for 15 seconds to inactivate endogenous alkaline phosphatase. PCR was performed, as described previously,[7] by incubation of the sections with 50 mL of 1× reaction buffer (Gibco, BRL), 1.5 U of *Taq* polymerase, 2 mmol/L of magnesium chloride, 40 mmol/L of deoxynucleoside triphosphates, 0.2 mmol/L of deoxyuridine triphosphate labeled with digoxigenin (Boehringer Mannheim, Lewes, England), and 60 pg each of *M tuberculosis* primers for the IS*6110* insertion sequence, which is specific for the *M tuberculosis* complex.[14]

The primer sequences were 5′-CCT GCG AGC GTA GGC GTC GG-3′ and 5′-CTC GTC CAG CGC CGC TTC GG-3′. The slides were sealed by using an assembly tool (Perkin Elmer, Cambridge, England) and placed in a thermocycler (Hybaid, Ashford, England). The program consisted of denaturation at 95°C for 1 minute, annealing at 70°C for 1 minute, and extension at 72°C for 1 minute, for 35 cycles. PCR products were detected by using alkaline phosphatase–conjugated sheep antibodies against antidigoxigenin (Boehringer Mannheim) diluted 1/500. The chromogen was 5-bromo-4-chloro-3-3-indolyl phosphate toluidine salt tetrazolium nitroblue (Boehringer Mannheim) diluted 1/50. Sections were counterstained with nuclear fast red to avoid any interference with the blue signal generated by mycobacterial DNA in the in situ PCR. Tissue sections from patients with pulmonary TB were used as positive control samples and previously identified negative samples as negative controls. To control for false-positives due to DNA repair, sections were subjected to PCR without *Taq* polymerase.

Conventional Tube PCR

Two sections of 5 μm each were used for conventional PCR, according to a protocol described for identifying mycobacterial DNA in archaeological specimens in which extraction and amplification present difficulties similar to those in fixed tissues.[15] Stringent precautions were taken against cross-contamination. Multiple sample blanks were used for negative controls during the DNA extraction, and water blanks were included in PCR amplifications to ensure there was no contamination. Positive control samples were not used in conventional PCR to avoid cross-contamination. The in situ PCR results were compared with those of conventional PCR and with the pathologic changes seen in the tissue.

Statistical Analysis

The Fisher exact test was used to compare the results of in situ and conventional PCR in TB and CD.

Results

In Situ PCR

Of the TB cases, 6 showed positive staining with in situ PCR: 3 at sites of granulomatous inflammation, 2 in inflammatory granulation tissue lining ulcers **Image 1**, and 1 in the surface epithelium and underlying lamina propria **Image 2** in foci with no evidence of granulomatous inflammation **Table 1**. The cells showing positive staining in granulomas and granulation tissue resembled macrophages.

Of 20 cases of CD, 1 showed focal positive staining with in situ PCR. Positivity was seen in macrophages within a granuloma **Image 3**. Endoscopy revealed that the cecum of this patient had been grossly deformed with multiple polypoid lesions. The mucosal biopsy specimen had shown small, noncaseating granulomas suggestive of CD, and positive staining with in situ PCR had been localized to one of these granulomas in the cecal mucosa. A review of the records revealed a positive response to treatment with sulfasalazine and a weight gain of 7 kg during a year, confirming the clinical diagnosis of CD.

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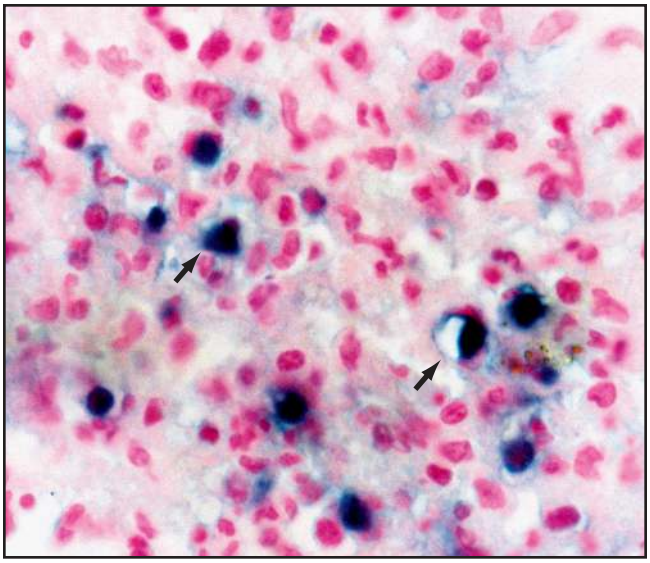

Image 1 In situ polymerase chain reaction stain for *Mycobacterium tuberculosis* showing focal cytoplasmic positive staining (arrows) in granulation tissue at a site without obvious granulomatous inflammation but lining an ulcer in the colonic mucosa of a patient with tuberculosis (×1,000).

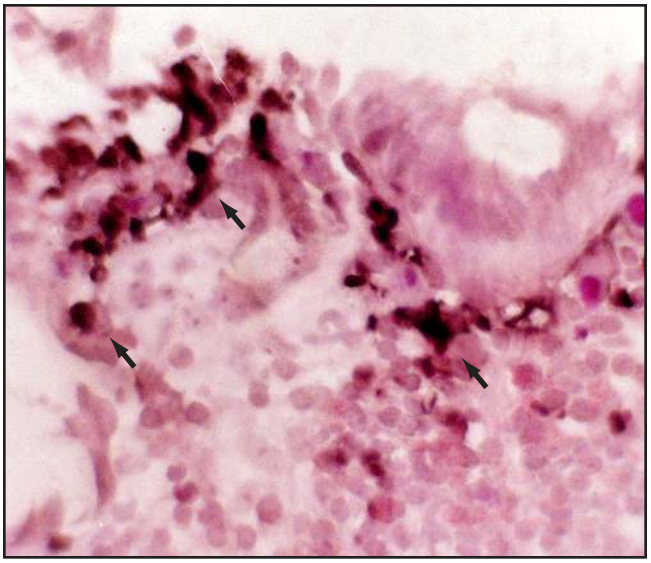

Image 2 In situ polymerase chain reaction stain for *Mycobacterium tuberculosis* showing positive staining in the surface epithelium and in cells lying in superficial lamina propria (arrows) of the colonic mucosa of a patient with tuberculosis. There is no evidence of granulomatous inflammation in this biopsy specimen (×400).

Tube PCR

Of the 20 TB cases, 5 were positive for *M tuberculosis* using conventional PCR, of which 3 were also positive with in situ PCR (Table 1). Of the 20 cases of CD, 1 was positive with conventional PCR only. Endoscopy revealed that the patient had ileal aphthous ulcers and segmental colitis. The biopsy had shown classic features of CD, including chronic active ileitis with focal enhancement, small granulomas, and microgranulomas. Review of the records showed a history of response to therapy for CD during a period of 1 year, confirming the diagnosis.

The difference in the incidence of positivity in TB and CD was not statistically significant, as expected owing to low numbers, for in situ or conventional PCR.

Correlation of Histologic Findings With In Situ PCR Staining

Tuberculosis

Caseation or acid-fast bacilli, the classic histologic features of TB, were seen in 9 of 20 cases. Of these, 4 were positive with in situ or conventional PCR, 2 with in situ and conventional PCR, 1 with in situ PCR alone, and 1 with conventional PCR only (Table 1). Among the cases positive with in situ PCR, staining was found in an area of granulomatous inflammation in 1 case and in granulation tissue lining ulcers without obvious granulomatous inflammation in the other 2 cases.

In 10 cases, there were histologic features suggestive but not diagnostic of TB, namely, large, confluent, or multiple granulomas without caseation or acid-fast bacilli. Three of these cases showed positive staining with in situ PCR at sites of granulomatous inflammation.

Table 1
Correlation of In Situ and Conventional PCR Results With Histologic Features in Tuberculosis

Case No.	Acid-Fast Bacilli	Caseation	In Situ PCR	Tube PCR
1	–	+	+	+
2	+	+	–	–
3	–	+	–	–
4	+	+	–	–
5	+	+	+	–
6	+	–	–	–
7	–	–	–	+
8	+	–	–	+
9	–	–	–	–
10	–	–	+	+
11	–	–	–	–
12	–	–	+	–
13	–	–	+	–
14	–	–	–	–
15	+	+	+	+
16	+	–	–	–
17	–	–	–	–
18	–	–	–	–
19	–	–	–	–
20	–	–	–	–

PCR, polymerase chain reaction; +, positive; –, negative.

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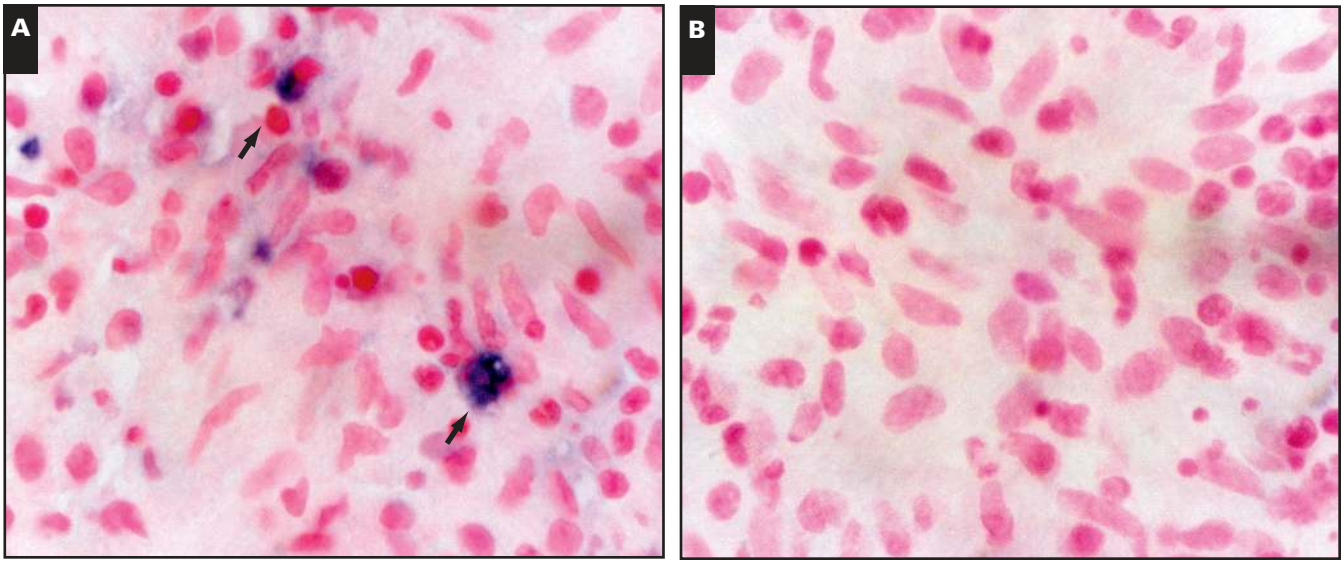

Image 3 **A**, In situ polymerase chain reaction stain for *Mycobacterium tuberculosis* showing cytoplasmic positive staining in cells (arrows) within an area of obvious granulomatous inflammation as demonstrated by the typical nuclei of epithelioid histiocytes, from the colonic mucosa of a patient with Crohn disease (×1,000). **B**, Negative control with no primers (×1,000).

Only 1 clinically diagnosed case of TB from our study did not show any of the histologic features that distinguish TB from CD. This case was negative with in situ and tube PCR.

Crohn Disease

Granulomas were seen in 14 of 20 cases of CD studied. One of these cases was positive with in situ PCR for *M tuberculosis,* and one was positive with tube PCR.

Discussion

Our study using in situ PCR showed the presence of *M tuberculosis* DNA in 6 of 20 mucosal biopsy specimens from patients with intestinal TB and 1 of 20 biopsy specimens from patients with CD. In TB, positivity was found in sites with and without granulomatous inflammation. In CD, positive staining was localized to a granuloma. We believe this is the first study to demonstrate *M tuberculosis* DNA within a granuloma of a patient with CD.

Earlier studies have reported the presence of *M tuberculosis* DNA in tissue without granulomas,[7,16,17] but these were not clinically proven cases of TB. A recent study used in situ PCR to demonstrate the presence of *M tuberculosis* DNA in adipose tissue from different extranodal locations in people with no clinical features of active TB.[18] Our findings suggest that even in the absence of granulomatous inflammation, in situ PCR may be helpful in making the diagnosis of TB on small biopsy specimens. The increasing incidence of CD in countries like India with a high prevalence of intestinal TB[2,3,13] poses a diagnostic challenge of increasing clinical significance that histologic and microbiologic studies available at present cannot always meet. It is possible that in situ PCR has the potential to be helpful in at least a subset of these difficult biopsy specimens in which TB and CD cannot be differentiated.

The low sensitivity of in situ PCR in our study could have had various causes. The extremely small quantity of tissue available in mucosal biopsy specimens and the limited number of sections used for DNA extraction may have been responsible for the presence of only low copy numbers of *M tuberculosis* DNA at the start of these experiments. The use of archival material is also known to decrease the yield in PCR.[19] The recovery of DNA from fixed tissue is related to the extent of penetration of formalin and the length of time of exposure to formalin. Although exposure time to formalin was limited to 12 hours, the biopsy specimens were extremely small, and some false-negative findings are likely.[19,20] The presence of PCR inhibitors at sites of extrapulmonary disease is another potential source of difficulty that may have caused low sensitivity.[20] Another study on intestinal biopsy specimens had a success rate similar to ours, with only 21.6% of intestinal TB cases being positive for *M tuberculosis* with conventional PCR.[13]

Among our CD biopsy specimens, positive staining was seen with in situ PCR in 1 case and with conventional tube PCR in 1 case. Because positive staining with in situ PCR was found within a granuloma, this is unlikely to have been a focus of false positivity. In addition, the PCR negative control samples were satisfactory. The antigens, DNA, or RNA of various infectious agents such as *M avium* subsp *paratuberculosis,*[8,21] *Y pseudotuberculosis,*[10] and measles

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virus[9] have been found in the tissue and granulomas in the intestinal wall of patients with CD. The present study is, however, the first to localize *M tuberculosis* DNA to a granuloma in patients with CD.

As with all other infectious agents identified in CD, it is possible that the presence of *M tuberculosis* is simply an epiphenomenon, owing to its wide prevalence in the population and incidental entry into the injured gut from contaminated food or water. It could also be postulated, however, that the mycobacterial DNA acts as a trigger for the abnormal inflammatory response seen. Mycobacteria are known to survive within granulomas in latent TB,[22-24] and with the high incidence of TB in India, it is possible that some of the granulomas seen in patients with CD in this population may be foci of latent TB. The presence of cell wall–defective tubercle bacilli could also account for PCR positivity in the absence of overt clinical features of TB.[22,25] The clinical response to therapy for CD and histologic features suggestive of CD rather than TB in these cases suggest, however, that the *M tuberculosis* DNA present is not contributing directly to the pathology or clinical manifestations.

The sensitivity of in situ PCR for *M tuberculosis* needs to be improved and studies done on larger numbers of cases of CD and TB before its usefulness in intestinal disorders is established. Using thicker or greater numbers of sections to increase the amount of tissue examined is a suggestion for improving sensitivity. Avoidance of formalin fixation would be ideal, but this requires cryostat facilities. Modifications in the PCR protocol, such as increasing the number of cycles,[26] increasing the amount of *Taq* polymerase,[27] or using real-time PCR[28] could also be useful. The role of *M tuberculosis* DNA in the pathology and pathogenesis of CD needs to be further explored.

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