

Fluorescent quantitative PCR of *Mycobacterium tuberculosis* for differentiating intestinal tuberculosis from Crohn's disease

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Abstract

Intestinal tuberculosis (ITB) and Crohn's disease (CD) are granulomatous disorders with similar clinical manifestations and pathological features that are often difficult to differentiate. This study evaluated the value of fluorescent quantitative polymerase chain reaction (FQ-PCR) for *Mycobacterium tuberculosis* (MTB) in fecal samples and biopsy specimens to differentiate ITB from CD. From June 2010 to March 2013, 86 consecutive patients (38 females and 48 males, median age 31.3 years) with provisional diagnoses of ITB and CD were recruited for the study. The patients' clinical, endoscopic, and histological features were monitored until the final definite diagnoses were made. DNA was extracted from 250 mg fecal samples and biopsy tissues from each patient. The extracted DNA was amplified using FQ-PCR for the specific MTB sequence. A total of 29 ITB cases and 36 CD cases were included in the analysis. Perianal disease and longitudinal ulcers were significantly more common in the CD patients ($P < 0.05$), whereas night sweats, ascites, and circumferential ulcers were significantly more common in the ITB patients ($P < 0.05$). Fecal FQ-PCR for MTB was positive in 24 (82.8%) ITB patients and 3 (8.3%) CD patients. Tissue PCR was positive for MTB in 16 (55.2%) ITB patients and 2 (5.6%) CD patients. Compared with tissue FQ-PCR, fecal FQ-PCR was more sensitive ($\chi^2 = 5.16$, $P = 0.02$). We conclude that FQ-PCR for MTB on fecal and tissue samples is a valuable assay for differentiating ITB from CD, and fecal FQ-PCR has greater sensitivity for ITB than tissue FQ-PCR.

Key words: Intestinal tuberculosis; Crohn's disease; Diagnosis; Fluorescent quantitative PCR; *Mycobacterium tuberculosis*

Introduction

Intestinal tuberculosis (ITB) and Crohn's disease (CD) are chronic granulomatous disorders (1) with similar clinical presentations and pathologies. Currently, the annual incidence of tuberculosis (TB) in China is approximately 1.3 million cases (second only to India) (2) and accounts for 14.3% of worldwide TB cases. The incidence of ITB, a common form of extrapulmonary TB, has increased in parallel with the overall resurgence of TB. In China, the prevalence of inflammatory bowel disease (IBD) has increased rapidly in recent years, mimicking its rapid growth in the developed world. The total number of IBD cases increased approximately 2.5-fold over the previous decade, and there has been a 15.7-fold increase in the number of CD patients (3). Misdiagnosis of CD and ITB is common in developing countries (4), particularly China (5). In the case of ITB misdiagnosis, unnecessary anti-TB

therapy poses a risk of toxicity and delays the treatment of CD. Conversely, treating ITB with steroids alone can lead to severe deterioration, or even death.

It is challenging to distinguish CD from ITB, particularly in highly endemic TB areas (6-8). A variety of clinical, endoscopic, and radiological criteria have been recommended to differentiate between the two conditions, but these criteria have demonstrated limitations (9-11). Some researchers have attempted to find new and specific differential diagnostic methods to distinguish between these conditions. T-SPOT.TB, an interferon-gamma release assay of whole blood, is a valuable assay for differentiating ITB from CD, particularly for the diagnostic exclusion of ITB based on its high specificity and negative predictive value (12-14). However, in highly endemic TB areas, such as China, where latent infection is widespread,

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a positive T-SPOT.TB result may not necessarily discriminate active TB from latent TB.

The TB polymerase chain reaction (PCR) assay is an interesting and promising approach. Amarapurkar et al. (15) reported that using TB PCR for biopsy specimens was a valuable method to differentiate ITB from CD. However, the positive rate of TB PCR was low (21.6%) in ITB patients. If the sensitivity is improved, *in situ* PCR is potentially useful for differentiating between ITB and CD (16). Ramadass et al. (17) have shown that, when combined with an acid-fast bacilli (AFB) culture, conventional fecal PCR, targeting the IS6110 sequence of the *Mycobacterium tuberculosis* (MTB) genome, can distinguish between ITB and CD. It remains to be determined whether the ITB diagnostic sensitivity of the test will increase with increasing refinement of PCR techniques.

In the present study, we evaluated the usefulness of fluorescent quantitative (FQ)-PCR in fecal samples and biopsy specimens as a means of differentiating between ITB and CD.

Material and Methods

From June 2010 to March 2013, 86 consecutive patients (38 females and 48 males, median age 31.3 years) with provisional diagnoses of ITB and CD were recruited into the study. The patients who had received treatment for TB in the 3 months prior to the sample collection were excluded. A single stool sample was collected from each patient and stored at -50°C prior to processing. All patients had undergone colonoscopies. Multiple biopsy specimens were obtained from affected areas and stored at -50°C prior to processing. The patients were investigated following routine clinical, endoscopic, and histological protocols and followed up after the therapy. All research involving human participants was approved by the Zhejiang Province People's Hospital. Written informed consent was obtained from the participants, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

The ITB diagnosis was established if at least one of the following criteria was met: 1) histological evidence of a caseating granuloma, 2) histological demonstration of AFB, 3) intestinal granulomatous inflammation accompanied by histologically or microbiologically confirmed extraintestinal TB, and 4) a positive MTB culture. The CD diagnosis was based on well-established clinical, endoscopic, radiological, and histological parameters in accordance with the criteria in the literature (18,19). In patients for whom the differentiation between ITB and CD was uncertain, anti-TB therapy was attempted for 2-3 months, and the final diagnosis was made based on the clinical and endoscopic response to the anti-TB therapy. The clinical response was determined by the loss of subjective symptoms. The endoscopic response was determined by the disappearance of ulcerations.

DNA was extracted from 250 mg fecal and biopsy tissues using a QiaAMP Stool DNA mini kit and QiaAMP DNA micro kit (Qiagen, Germany), respectively. The specific sequence was amplified using an MTB DNA (PCR-fluorescence) diagnostic kit with Roche LightCycler PCR amplification (Roche, Germany). The FQ-PCR mixture was prepared in a total volume of 20 μL , which contained 2- μL DNA sample. FQ-PCR was performed under the following conditions: initial activation at 93°C for 2 min, followed by 40 cycles at 93°C for 5 s, 57°C for 45 s, and, finally, extension at 72°C for 1 s. Amplifications were performed and results analyzed according to the manufacturer's instructions.

Statistical analysis

The differences in proportions were analyzed using the Fisher exact test. The Student *t*-test was used to compare mean values of the appropriate parameters in the two groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

Eighty-six patients were initially recruited for the collection of fecal samples and biopsy tissues. Twenty-one patients were excluded from the study because of diagnoses other than ITB or CD. A total of 29 ITB cases and 36 CD cases were included in the analysis.

The clinical, endoscopic, and histological features differentiating between ITB and CD are demonstrated in Table 1. Perianal disease and longitudinal ulcer were significantly more common in the CD patients than in the ITB patients, whereas night sweats, ascites and circumferential ulcers were significantly more common in the ITB patients than in the CD patients. Stricture was more frequently observed in the CD patients than in the ITB patients; however, the difference did not reach statistical significance.

The results of fecal TB FQ-PCR, tissue TB FQ-PCR, and the tuberculin skin test (TST) are shown in Table 2. Fecal TB FQ-PCR was positive in 24 (82.8%) ITB patients and 3 (8.3%) CD patients. Tissue FQ-PCR was positive in 16 (55.2%) ITB patients and 2 (5.6%) CD patients. Compared to tissue TB FQ-PCR, fecal TB FQ-PCR had greater sensitivity ($X^2 = 5.16$, $P = 0.02$). Tissue TB FQ-PCR had a sensitivity similar to that of the TST.

The overall sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of fecal TB FQ-PCR, tissue TB FQ-PCR, and TST are listed in Table 3. Fecal TB FQ-PCR had greater sensitivity and NPV than tissue FQ-PCR. Tissue TB FQ-PCR had greater specificity and PPV than the TST.

Discussion

The symptoms and signs of ITB are nonspecific and share many similarities with CD. There are many

Table 1. The differentiating clinical, endoscopic, and histological features of patients with intestinal tuberculosis (ITB) and Crohn's disease (CD).

	ITB (n=29)	CD (n=36)
Clinical presentation		
Age, years (means \pm SD)	30.3 \pm 11.6	31.7 \pm 12.5
Gender (male:female)	14:15	22:14
Abdominal pain	23 (79.3%)	33 (91.7%)
Diarrhea	19 (65.5%)	27 (75.0%)
Hematochezia	6 (20.7%)	12 (33.3%)
Ascites	8 (27.6%)	0 (0.00%)*
Fever	17 (58.6%)	18 (50.0%)
Night sweats	16 (55.2%)	8 (22.2%)*
Perianal disease	1 (3.4%)	8 (22.2%)*
Endoscopic features		
Longitudinal ulcer	4 (13.8%)	13 (36.1%)*
Circumferential ulcer	18 (62.1%)	6 (16.7%)*
Aphthous ulcers	3 (10.3%)	9 (25.0%)
Cobblestone appearance	5 (17.2%)	12 (33.3%)
Pseudo-polyps	8 (27.6%)	16 (44.4%)
Stricture	4 (13.8%)	12 (33.3%)*
Histological features		
Caseating granulomas	3 (10.3%)	0 (0.00%)
Non-caseating granulomas	9 (31.0%)	5 (13.9%)
Lymphocyte aggregation	12 (41.4%)	12 (33.3%)

Data are reported as number with percent in parentheses. *P<0.05, compared to ITB (Fisher exact test).

proposed clinical, radiological, endoscopic, and histological features that distinguish ITB from CD, including a prolonged illness, diarrhea, hematochezia, weight loss, extraintestinal manifestations, fever, ascites, deep linear ulcers, cobble-stone appearance, involvement of the sigmoid colon, coexisting pulmonary lesions, and abdominal lymphadenopathy, etc. (20-22). In the present study, perianal disease and longitudinal ulcers were more common in the CD patients, whereas night sweats, ascites, and circumferential ulcers were more common in the ITB patients. In general, positive findings in the histology and microbiology of ITB and CD are unsatisfactory (6), and a comprehensive analysis of the clinical, endoscopic, and histological disease features may help to

confirm the diagnosis. Li et al. (23) reported that a combination of clinical and endoscopic parameters leads to elevated accuracy and specificity in differentiating CD from ITB. Further research involving a large sample and multicenter cooperation is needed.

The most reliable differentiation method is finding evidence of MTB in intestinal tissues. Unfortunately, AFB staining has low sensitivity. In addition, the biopsy culture for MTB is time consuming (3-8 weeks), and the results are frequently negative (accuracy ranging from 25 to 35%) (11). The PCR assay provides a rapid and sensitive approach, and PCR techniques are being refined to increase their diagnostic sensitivity. FQ-PCR has become a potentially powerful alternative in microbiological diagnosis because of its simplicity, rapidity, reproducibility, and accuracy. Hillemann et al. (24) have shown that the real-time PCR assay exhibits a higher sensitivity (66.7%) for detecting MTB complex DNA than an alternative in-house IS6110 PCR (33.3%) in formalin-fixed, paraffin-embedded tissues.

The PCR detection of mycobacteria in histopathological specimens was introduced as a rapid and useful technique for diagnosing pulmonary and extrapulmonary TB (25,26). Gan et al. (27) reported a 64.1% (25/39) positivity rate from PCR in 39 ITB biopsy specimens, but zero positivity from PCR in 30 CD specimens. A study using *in situ* PCR indicated the presence of MTB DNA in 6 of 20 mucosal biopsy specimens from patients with ITB and in 1 of 20 biopsy specimens from patients with CD (16). In the present study, PCR detection of mycobacteria in biopsy specimens was positive in 16 (55.2%) ITB cases and 2 (5.6%) CD cases. Thus, tissue FQ-PCR is potentially useful to differentiate between ITB and CD. Compared to fecal FQ-PCR, tissue FQ-PCR has a lower sensitivity. In this study, all tissue samples were obtained from endoscopic biopsies, and the tissue samples were superficial and small in number. MTB would not be distributed evenly in the tissue. Low numbers of extracted MTB DNA in mucosal biopsy specimens could be responsible for the false-negative results of tissue PCR. In the study, although the tissue TB FQ-PCR assay had a sensitivity similar to the TST, tissue TB FQ-PCR had higher specificity and PPV than the TST. This finding suggests that tissue TB FQ-PCR has more clinical value than the TST.

Table 2. Results of fecal FQ-PCR, tissue FQ-PCR and TST for *Mycobacterium tuberculosis* in the patient groups.

	Intestinal TB (%)	Crohn's disease (%)	P
Total number of patients	29	36	
Fecal FQ-PCR positive	24 (82.8%)	3 (8.3%)	0.00
Tissue FQ-PCR positive	16 (55.2%)	2 (5.6%)	0.00
TST positive	17 (58.6%)	11 (30.6%)	0.02

Data are reported as number with percent in parentheses. FQ-PCR: fluorescent quantitative polymerase chain reaction; TST: tuberculin skin test. The Fisher exact test was used for statistical analyses.

Table 3. Clinical evaluation of fecal FQ-PCR, tissue FQ-PCR and TST.

	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
Fecal FQ-PCR	82.8% (65.45-92.4)	91.7% (78.2-97.1)	88.9% (71.9-96.2)	86.8% (72.7-94.3)
Tissue FQ-PCR	55.2% (37.6-71.6)	94.4% (81.9-98.5)	88.9% (67.2-96.9)	72.3% (58.2-83.1)
TST	58.6% (40.7-74.5)	69.4% (53.0-83.1)	60.7% (42.4-76.5)	67.6% (51.38-80.45)

FQ-PCR: fluorescent quantitative polymerase chain reaction; TST: tuberculin skin test; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value.

Unlike an endoscopic biopsy, fecal PCR is noninvasive and less subject to sampling errors. Fecal PCR can theoretically detect mycobacterial DNA shed from anywhere along the length of the gastrointestinal tract. Balamurugan et al. (28) reported that the sensitivity, specificity, PPV, and NPV of PCR detection of MTB in fecal samples of ITB patients were 88.8, 100, 100, and 93.7%, respectively. Further studies have shown that fecal PCR combined with AFB culture provides a good test to distinguish between ITB and CD, with a sensitivity of 95% and specificity of 88% (17). In the present study, fecal FQ-PCR was positive in 24 (82.8%) ITB cases and 3 (8.3%) CD cases. The sensitivity, specificity, PPV, and NPV of fecal FQ-PCR were 82.8, 91.7, 88.9, and 86.8%, respectively. These results suggest that fecal FQ-PCR is a valuable assay for differentiating ITB from CD. However, we did not find that fecal FQ-PCR detection of MTB was superior to regular PCR (28).

The results showed that fecal PCR was positive in

three (8.3%) CD cases and tissue PCR was positive in two (5.6%) CD cases. The presence of MTB may be an epiphenomenon, considering its high prevalence in the general population and incidental entry into the inflamed bowel from contaminated food or water. Latent TB infection may exist in patients with CD, which may be unmasked by infliximab therapy (29,30). A series of studies using genetic and serological techniques now purport to have identified mycobacteria at a higher prevalence in Crohn's patients than in control subjects (31-33). However, whether mycobacteria in CD act as a trigger for the abnormal inflammatory response remains an issue for further research.

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