Utility of in vitro interferon-γ release assay in differential diagnosis between intestinal tuberculosis and Crohn’s disease

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OBJECTIVE: To evaluate the diagnostic utility of interferon-γ release assay (T-SPOT.TB) for the differential diagnosis between Crohn’s disease (CD) and intestinal tuberculosis (ITB).

METHODS: A total of 103 CD and 88 ITB patients, confirmed by histology and anti-tuberculosis treatment response from 2003 to 2011, were included. Their characteristics and clinical features were recorded. Mycobacterium tuberculosis (MTB) polymerase chain reaction (PCR) of IS6110, in vitro T-SPOT.TB, tuberculin skin test (TST), immunoglobulin G (IgG) antibody to MTB (protein chip), serum anti-Saccharomyces cerevisiae antibodies (ASCA IgG, chronic inflammatory bowel disease profile) and acid-fast staining of biopsied colonic tissue specimens were performed. Statistical analysis was conducted to determine their concordance with the diagnosis and its sensitivity, specificity, positive (PPV) and negative predictive value (NPV).

RESULTS: Abnormal pulmonary X-ray, ascites and lesions of both cecum and ascending colon were more associated with ITB, while intestinal surgery and lesions of both ileum and adjacent colon were more commonly seen in CD. Significant diagnostic concordance was found using T-SPOT.TB ($k = 0.786$) by consistency test. The sensitivity, specificity, PPV and NPV of T-SPOT.TB were 86%, 93%, 88% and 91%, respectively, and the sensitivity and NPV were significantly higher than other examinations ($P < 0.05$).

CONCLUSION: T-SPOT.TB is a valuable assay in differentiating ITB from CD, particularly in the diagnostic exclusion of ITB based on its high specificity and NPV.

KEY WORDS: Crohn disease, intestinal tuberculosis, Mycobacterium tuberculosis, polymerase chain reaction.

INTRODUCTION

Intestinal tuberculosis (ITB) and Crohn’s disease (CD) are both characterized by chronic granulomatous inflammation, and it is often difficult to differentiate between CD and ITB in clinical practice. CD is considered to be a disease with yet unknown etiology and occurs as a result of innate dysfunctional immune response and subsequent mucosal inflammation in susceptible individuals.1
In 2010 the countries with the highest incidence of tuberculosis (TB) were India (2.0–2.5 million) and China (0.9–1.2 million). India alone accounted for 26% of all TB cases worldwide, while China and India altogether accounted for 38%. There is still no adequate identification of active disease and drug resistance, and TB has not yet been effectively controlled. The latter is further complicated due to probable human immunodeficiency virus (HIV) co-infection and widespread population migration. Intestinal involvement is common for extrapulmonary TB, following TB in the lymph nodes and peritoneum, and is particularly difficult to diagnose, especially in areas with high incidence of TB. Hence, it is quite important to obtain a definite diagnosis as TB treatment protocols are quite different from CD.

The differentiation of the two diseases remains a challenge because they present with similar symptoms, and conventional clinical, laboratory, endoscopic, radiological and even histopathological examinations cannot effectively distinguish between them. Several studies have demonstrated that the misdiagnosis rate between CD and ITB is up to 50–70%. Furthermore, following the use of steroids, immunosuppressants and biological agents after a presumptive diagnosis of CD, severe and sometimes fatal complications such as systemic dissemination of TB can also lead to misdiagnosis.

In daily clinical practice, various methods including tuberculin skin test (TST), traditional Mycobacterium tuberculosis (MTB) culture and acid-fast bacilli staining are used for the detection of TB infection. However, the low sensitivity and specificity and complicated process of samples limited the use of these methods. In some reports, polymerase chain reaction (PCR) assay was in particular associated with high sensitivity but low specificity. Currently, endoscopic and histopathological examinations are conducted to differentiate between the two disorders, but specific and precise criteria are still lacking.

T-SPOT.TB test is a commercial blood interferon (IFN)-γ assay measuring the number of activated T cells by identifying IFN-γ release when stimulated by MTB-specific antigens, including early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). In the present study, we aimed to compare five laboratory examinations for the detection of MTB infection, in particular IS6110 PCR and T-SPOT.TB test, and to evaluate the utility of T-SPOT.TB for differentiating between CD and ITB.

PATIENTS AND METHODS

Patients

A total of 103 CD patients and 88 ITB patients were enrolled from the Inflammatory Bowel Disease Center of Zhongnan Hospital from 2003 to 2011. During their initial clinical consultation in the Department of Gastroenterology, medical history of each patient was recorded and routine tests were conducted including TST, immunoglobulin G (IgG) antibody to MTB (Protein Chip Diagnostic kit for IgG antibody to MTB; Nanjing Potomac Biotechnology, Nanjing, Jiangsu Province, China), anti-Saccharomyces cerevisiae antibodies (ASCA) IgG (chronic inflammatory bowel disease profile; EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) and acid-fast staining. Before the therapy, some patients also underwent coloscopic examination and specimens from the lesions were obtained for histological examination and MTB DNA isolation. T-SPOT.TB test is started 2 years ago in our hospital, therefore, only few included patients underwent this test. In these patients, 5 ml peripheral blood was collected in tubes with heparin lithium and processed within 4 h.

The diagnosis of CD was made according to the World Health Organization (WHO) diagnostic criteria based on clinical, endoscopic, radiological and pathological features. The diagnosis of ITB was made according to the following criteria: (i) identification of MTB by acid-fast staining or culture in biopsied specimens; (ii) presence of caseating granulomas in histological examination or (iii) improvement of clinical and endoscopic disease activity after at least 3 months of anti-TB therapy.

The study protocol was approved by the Ethics Committee of Wuhan University Zhongnan Hospital.

DNA extraction

A total of 74 fresh colonic mucosal tissue specimens were taken during endoscopic examination and were subsequently stored at −20°C till assayed. After homogenization with 300 μl of sodium-tris-ethylenediaminetetraacetate (STE) buffer, consisting of 50 mmol/L Tris-HCL (pH 8.6), 10 mmol/L ethylenediaminetetraacetate (EDTA), 1% sodium dodecyl sulfate and 20 μl proteinase K (20 mg/mL), the suspension was incubated at 55°C for 12–16 h. The suspension was boiled for 10 min and then phenol-chloroform extraction was carried out. DNA was

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dissolved completely in 50 μL TE buffer (10 mmol/L Tris-HCl + 1 mmol/L EDTA, pH 8.0) and stored at -20°C till further assayed.

Altogether 117 paraffin-embedded tissue specimens were sliced with the thickness of 8 μm for each block, and were placed in 1.5 mL of eppendorf tubes, and were then cleaned with xylene, absolute ethanol, 75% ethanol and 50% ethanol for 30 min, respectively. The remaining procedure was the same with that for the fresh tissue specimens.

**IS6110 amplification of MTB DNA**

Insertion segment IS6110 (GenBank: DQ217928) is a 1.35-kb sequence found only in the MTB complex. The primers used to amplify a fragment of 123 bp were: forward, 5′-CTGTGACGTAGGCG TCGG-3′ (P1) and reverse, 5′-CTCGTCCAGCGCCGCTTCGG-3′ (P2), as recommended by Eisenach et al. The 50 μL mixture was processed as follows: initial denaturation at 95°C for 5 min, then denaturation for 35 cycles at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min, and finally extension at 72°C for 7 min. The product was separated by electrophoresis with 2% agarose gel and observed under ultraviolet. Positive and negative controls were provided using MTB H37Rv DNA and double-distilled water.

PCR-positive product was digested with restriction enzyme Sal I (MBI Fermentas, Hanover, MD, USA) at 37°C for 12–16 h. The 123 bp fragment was replaced by 53 bp and 70 bp fragments, and confirmed by 12% polyacrylamide gel electrophoresis. Segments of products were sequenced (Shanghai Map Biotech, Shanghai, China) to give additional confirmation.

All the DNA samples were tested for human β-globin gene sequence to ensure they there were no inhibitors. The forward and reverse primers were: 5′-GAAGAGCC AAGGACAGGTAC-3′ (P1) and 5′-CAACTTCATCCA CGITGCAC-3′ (P2), as reported in the literature. Finally, a 268 bp fragment was amplified and identified by electrophoresis using 2% agarose gel.

**T-SPOT.TB assay**

Peripheral venous blood (5 mL) collected in tubes with heparin lithium was obtained from each patient enrolled in the most recent 2 years (67 CD and 42 ITB) and processed within 4 h. According to the instructions, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (GE Healthcare, Little Chalfont, UK) and washed twice in RPMI 1640 and AIM-V medium (Invitrogen, Carlsbad, CA, USA). Four wells were used for each sample: negative control, ESAT-6, CFP-10 and positive control. IFN-γ secreted by T cells was seen as spots, which were counted according to the instructions of the manufacturer.

**Related clinical laboratory examinations**

TST was performed in 161 patients, including 85 CD and 76 ITB. The development of induration of more than 10 mm was considered positive. Acid-fast staining assay was conducted by two pathologists on the paraffin-embedded tissues from 182 patients (99 CD and 83 ITB). IgG antibody to MTB including LAM, 38 kD and 16 kD was tested for 88 patients (38 CD and 50 ITB). ASCA IgG was determined for 116 patients (65 CD and 51 ITB) by the indirect immunofluorescent technique. All related clinical tests were performed in the clinical laboratory or the Department of Pathology at Wuhan University Zhongnan Hospital.

**Statistical analysis**

Sensitivity, specificity for active TB, positive (PPV) and negative predictive value (NPV), and their 95% confidence interval (CI) for the differential diagnosis of the two diseases were calculated using Microsoft Excel 2003. The concordance of the different laboratory methods was also evaluated by κ coefficients (κ > 0.75 excellent agreement; κ ≥ 0.4 and ≤0.75, good agreement; κ < 0.4, poor agreement). Patients’ characteristics, clinical features and the difference in the laboratory examinations between CD and ITB were compared using Student’s t-test, χ² test, Fisher’s exact test and binary logistic regression analysis, respectively, according to numbers and accounting data, with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

**RESULTS**

**Characteristics and clinical features of patients with CD and ITB**

Overall, 103 patients with CD and 88 patients with ITB were included in the study. Their characteristics and clinical features are shown in Table 1. Previous active pulmonary TB history (17.0%), abnormal pulmonary X-ray findings (47.7%), ascites (20.5%) were
more associated with ITB ($P < 0.05$), while extraintestinal manifestations (29.1%) including oral ulcer, erythema nodosum, conjunctivitis and joint diseases, intestinal surgery (42.7%) were more commonly seen in CD ($P < 0.01$). The location of lesions was different as ITB mostly involved the colon ($P < 0.05$).

The above six statistically different parameters were further analyzed using binary logistic regression analysis. As a result, the frequencies of abnormal pulmonary X-ray (OR 6.536, 95% CI 2.586–16.521), ascites (OR 4.788, 95% CI 1.108–20.698) and lesions at both cecum and ascending colon (OR 1.361, 95% CI 0.522–3.549) were more commonly seen in ITB, while intestinal surgery (OR 0.157, 95% CI 0.064–0.385), lesions at both ileum and adjacent colon (OR 0.340, 95% CI 0.146–0.792) were more significant for the diagnosis of CD.

### Diagnostic value of IS6110, T-SPOT.TB, acid-fast staining, protein chip and ASCA IgG of patients with CD and ITB

The positive rates of IS6110 PCR, T-SPOT.TB, and other parameters of patients with CD and ITB are shown in Table 2. In the IS6110 PCR examination, 102 DNA samples from 117 paraffin-embedded tissues and the entire 74 DNA samples from fresh specimens were available, 7.3% of the patients with CD (7/96) and 57.5% with ITB (46/80) were positive ($P < 0.0001$). The electrophoregrams of IS6110 PCR before and after the Sal I enzyme digestion are shown in Figure 1. The protocol of T-SPOT.TB assay is shown in Figure 2. The value of T-SPOT.TB for the final diagnosis of ITB was found to be highly significant ($\kappa = 0.786$, 95% CI 0.598–0.974), IS6110 PCR and TST also provided good agreement ($\kappa = 0.517$ and 0.401, respectively) Combination of IS6110 PCR and T-SPOT.TB ($\kappa = 0.600$), T-SPOT.TB and TST ($\kappa = 0.519$), IS6110 PCR and TST ($\kappa = 0.430$), the agreement for ITB diagnosis was good. However, the diagnostic value of protein chip ($\kappa = 0.343$) and acid-fast staining ($\kappa = 0.117$) were poor.

### Diagnostic validity of IS6110 PCR, T-SPOT.TB, TST, acid-fast staining and protein chip

The sensitivity of T-SPOT.TB was 86% (95% CI 0.75–0.96), which was significantly higher than other examinations ($P < 0.05$); and its specificity for active TB disease was also high (93%) (Table 3). Its NPV was 91% (95% CI 0.84–0.98), which was also significantly higher than other examinations ($P < 0.05$). Combination of T-SPOT.TB, PCR and TST tests had the specificity and PPV of 100% and 100%, respectively, for the diagnosis of ITB; however, the sensitivity and NPV were only 31% and 72%. T-SPOT.TB combined with PCR had the sensitivity, specificity, PPV and NPV of more than 50%, even reached 100%.

### DISCUSSION

In recent years, due to the increasing incidence of CD and widespread and drug-resistant TB, it is important...
to differentiate between CD and ITB in order to provide effective and prompt therapies. So far, significantly reliable methods for the differential diagnosis have not been identified yet. Based on the clinical features in our study, ascites, intestinal surgery, pulmonary X-ray and the location of lesions were found to contribute to the differentiation between CD and ITB when analyzed using binary logistic regression. Conventional laboratory methods such as acid-fast staining have low sensitivity, and TB culture is time-consuming. In this study, IS6110 PCR (κ = 0.517), TST (κ = 0.401), and especially T-SPOT.TB (κ = 0.786) showed satisfactory performances in diagnosing or ruling out ITB, which were better than the protein chip and acid-fast staining.

Few studies have investigated the utility of T-SPOT.TB in differentiating CD and ITB. Richeldi et al.\(^{20}\) and Table 2. Results of IS6110 polymerase chain reaction (PCR), interferon (IFN)-γ release assay (T-SPOT.TB) and other parameters and concordance for differentiation between Crohn’s disease (CD) and intestinal tuberculosis (ITB)

<table>
<thead>
<tr>
<th></th>
<th>CD (N = 103), Positive/total (%)</th>
<th>ITB (N = 88), Positive/total (%)</th>
<th>P value value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SPOT.TB</td>
<td>5/67 (7.5)</td>
<td>36/42 (85.7)</td>
<td>&lt;0.0001* 0.786 (0.598–0.974)</td>
</tr>
<tr>
<td>IS6110 PCR</td>
<td>7/96 (7.3)</td>
<td>46/80 (57.5)</td>
<td>&lt;0.0001* 0.517 (0.377–0.657)</td>
</tr>
<tr>
<td>TST</td>
<td>17/85 (20.0)</td>
<td>46/76 (60.5)</td>
<td>&lt;0.0001* 0.401 (0.249–0.552)</td>
</tr>
<tr>
<td>Acid-fast staining</td>
<td>0/99 (0.0)</td>
<td>9/83 (10.8)</td>
<td>0.005* 0.117 (0.049–0.185)</td>
</tr>
<tr>
<td>IgG to MTB (protein chip)</td>
<td>11/38 (28.9)</td>
<td>32/50 (64.0)</td>
<td>0.001* 0.343 (0.137–0.549)</td>
</tr>
<tr>
<td>ASCA IgG</td>
<td>5/65 (7.7)</td>
<td>0/51 (0.0)</td>
<td>0.118 –</td>
</tr>
<tr>
<td>IS6110 PCR, T-SPOT.TB, TST</td>
<td>0/52 (0.0)</td>
<td>9/28 (32.1)</td>
<td>&lt;0.0001* 0.368 (0.202–0.543)</td>
</tr>
<tr>
<td>IS6110 PCR, T-SPOT.TB</td>
<td>0/52 (0.0)</td>
<td>15/28 (53.6)</td>
<td>&lt;0.0001* 0.600 (0.399–0.801)</td>
</tr>
<tr>
<td>IS6110 PCR, TST</td>
<td>1/52 (1.9)</td>
<td>11/28 (39.3)</td>
<td>&lt;0.0001* 0.430 (0.241–0.619)</td>
</tr>
<tr>
<td>T-SPOT.TB, TST</td>
<td>1/52 (1.9)</td>
<td>14/29 (48.3)</td>
<td>&lt;0.0001* 0.519 (0.321–0.716)</td>
</tr>
</tbody>
</table>

*P < 0.05, significant statistical difference. CI, confidence interval; TST, tuberculin skin test; IgG, immunoglobulin G; MTB, Mycobacterium tuberculosis; ASCA, anti-Saccharomyces cerevisiae antibodies.

Figure 1. IS6110 polymerase chain reaction (PCR) analysis. (a) IS6110 PCR amplification. Lane 1, Crohn’s disease (CD) sample; lane M, 100 bp DNA marker; lanes 2 and 3, intestinal tuberculosis (ITB) samples; lane 4, double-distilled water control; lane 5, Mycobacterium tuberculosis (MTB) H37Rv DNA. (b) Products of IS6110 PCR amplification digested by restriction enzyme Sal I. Lane M, 50 bp DNA marker; lane 1, MTB H37Rv DNA; lanes 2–6, positive PCR products of ITB and CD samples.

Figure 2. Interferon (IFN)-γ release assay (T-SPOT.TB) test. ESAT-6, early secretory antigenic target 6; CFP-10, culture filtrate protein 10; P, positive; N, negative.

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Tavast et al.\(^2\) have compared the concordance between three tests (QuantiFERON-TB Gold In-Tube test, TST and T-SPOT.TB), most of which showed good results even in the immunosuppressed patients such as those underwent liver transplantation or with hematological malignancies. However, when using for latent tuberculosis infection (LTBI), the diagnostic concordance between TST and T-SPOT.TB has been reported as moderate (\(\kappa = 0.47\) and 0.40, respectively), and even lower in patients with HIV infection (\(\kappa = 0.16\)).\(^2\)

Many studies have investigated the use of PCR in the differential diagnosis of ITB and CD.\(^12,13,22,23\) However, there has been no study comparing the utility of T-SPOT.TB and PCR in the two diseases till now. Balamurugan et al.\(^12\) found that the IS6110 PCR amplification of MTB in fecal samples had a sensitivity of 89% and specificity of 100% in the diagnosis of ITB. In a Chinese study, Gan et al.\(^23\) reported that positive rate by PCR in TB specimens was 64.1% (25/39) and 0% (0/30) in CD specimens. Another study, also using conventional PCR, showed that the PCR assay had high specificity (95%) for the diagnosis of ITB.\(^22\) In situ PCR and other PCR assays were used to differentiate the two disorders. An in situ PCR was positive in 6 out of 20 ITB and 1 out of 20 CD biopsied specimens, and the investigators concluded that in situ PCR is potentially useful to differentiate ITB from CD if the sensitivity can be improved.\(^13\) However, it is difficult to obtain optimal sensitivity since the characteristics of specimens, including the depth of biopsy and the sample storage period, may differ. Similar to our result, intestinal specimens from CD patients have been found to contain MTB, which may be due to latent infection or a consequence of mucosal inflammation caused by mucosal immune dysregulation.\(^13\)

Compared with other methods, T-SPOT.TB in our study showed significantly higher sensitivity (86%) for the diagnosis of ITB. In addition, the relatively high specificity in active disease (93%) and significantly high NPV (91%) indicate its clinical value for the exclusion of MTB infection. Our results were similar to other reports on the sensitivity of T-SPOT.TB (83–100%).\(^24,28\) However, the specificity, PPV and NPV have been shown to vary from 47–100%, 60–91% and 79–94.1%, respectively.\(^21,27,31\) In developed countries the specificity for active disease may be higher than that in high-incidence areas due to the prevalence of LTBI. Kang et al.\(^30\) demonstrated that the sensitivity of T-SPOT.TB for active pulmonary tuberculosis was 92%, which is similar to our result, but the specificity of 47% is much lower than that in our study (93%). This study was performed in South Korea where the prevalence of LTBI is similar to that in China,\(^32\) including the participants with suspected pulmonary TB; however, the patients in our study group included CD patients who are known to have a relatively low rate of TB infection. Moreover, our study showed that the sensitivity, specificity, PPV and NPV of T-SPOT.TB were all more than 85% in the differential diagnosis. Furthermore, combined T-SPOT.TB and PCR had the sensitivity of more than 50%, with NPV reached 80% and specificity and PPV of 100%. Hence, T-SPOT.TB assay and the combination of T-SPOT.TB and PCR were considered to be reliable in differentiating between CD and ITB, and for the exclusion of MTB colonization.

In several areas and countries including the United States, Europe and Japan, T-SPOT.TB assay has been mainly used to identify LTBI in patients with rheumatoid arthritis before the initiation of tumor necrosis.

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### Table 3. Diagnostic validity of IS6110 polymerase chain reaction (PCR), interferon-γ release assay (T-SPOT.TB) and other laboratory examinations

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, % (95% CI)</th>
<th>Specificity, % (95% CI)</th>
<th>PPV, % (95% CI)</th>
<th>NPV, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SPOT.TB</td>
<td>86 (0.75–0.96)(^*)</td>
<td>93 (0.86–0.99)</td>
<td>88 (0.78–0.98)</td>
<td>91 (0.84–0.98)</td>
</tr>
<tr>
<td>IS6110 PCR</td>
<td>58 (0.47–0.68)</td>
<td>93 (0.88–0.98)</td>
<td>87 (0.78–0.96)</td>
<td>72 (0.65–0.80)</td>
</tr>
<tr>
<td>TST</td>
<td>60 (0.49–0.71)</td>
<td>80 (0.71–0.89)</td>
<td>73 (0.62–0.84)</td>
<td>69 (0.60–0.78)</td>
</tr>
<tr>
<td>Acid-fast staining</td>
<td>11 (0.04–0.18)</td>
<td>100 (1.00–1.00)</td>
<td>100 (1.00–1.00)</td>
<td>57 (0.50–0.65)</td>
</tr>
<tr>
<td>Protein chip</td>
<td>64 (0.50–0.74)</td>
<td>71 (0.57–0.85)</td>
<td>74 (0.61–0.87)</td>
<td>60 (0.46–0.74)</td>
</tr>
<tr>
<td>IS6110 PCR, T-SPOT.TB, TST</td>
<td>31 (0.14–0.48)</td>
<td>100 (1.00–1.00)</td>
<td>100 (1.00–1.00)</td>
<td>72 (0.62–0.83)</td>
</tr>
<tr>
<td>IS6110 PCR, T-SPOT.TB</td>
<td>54 (0.35–0.72)</td>
<td>100 (1.00–1.00)</td>
<td>100 (1.00–1.00)</td>
<td>80 (0.70–0.90)</td>
</tr>
<tr>
<td>IS6110 PCR, TST</td>
<td>39 (0.21–0.57)</td>
<td>98 (0.94–1.00)</td>
<td>92 (0.76–1.00)</td>
<td>75 (0.65–0.85)</td>
</tr>
<tr>
<td>T-SPOT.TB, TST</td>
<td>48 (0.30–0.66)</td>
<td>98 (0.94–1.00)</td>
<td>93 (0.81–1.00)</td>
<td>77 (0.67–0.87)</td>
</tr>
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</table>

\(^*P < 0.05\), compared with other examinations.

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factor (TNF)-α antagonist therapy. In China, the most appropriate and relevant use of the test is still being evaluated.

Another assay using ESAT-6 and CFP-10 which is similar to T-SPOT.TB is QFT-G, which is a whole-blood IFN-γ test, and has similar specificity to T-SPOT.TB, but slightly lower sensitivity.\(^{33,34}\) According to recent studies,\(^{21,30}\) it may be possible to perform these assays directly on mononuclear cells isolated from the intestinal epithelium and to diagnose ITB in its early stage. New technology using ESAT-6 and CFP-10 such as single-stranded DNA (ssDNA) aptamer should be studied further.\(^{35}\) There are some limitations in our research. First, not every patient was subjected to all the methods. And second, the sample size was not large enough.

In conclusion, our study demonstrated that T-SPOT.TB is a useful assay which can be used as an adjunctive tool in the differentiation of ITB from CD, especially for the exclusion of ITB due to its high specificity and NPV.

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