Application value of tissue tuberculosis antigen combined with Xpert MTB/RIF detection in differential diagnosis of intestinal tuberculosis and Crohn's disease

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Research article

Keywords: Intestinal Tuberculosis, Crohn's disease, Xpert MTB/RIF, Antigen, Diagnosis

Posted Date: August 5th, 2019

DOI: https://doi.org/10.21203/rs.2.12435/v1

Version of Record: A version of this preprint was published at BMC Infectious Diseases on May 28th, 2021. See the published version at https://doi.org/10.1186/s12879-021-06210-8.

Abstract

Background: The purpose of this study was to evaluate the value of Xpert MTB/RIF detection and tuberculosis antigen detection of Mycobacterium tuberculosis (MTB) in intestinal tissues for differentiating intestinal tuberculosis (ITB) from Crohn's disease (CD). Materials and Methods: A total of 110 patients who were clinically diagnosed with CD or ITB between January 2016 and March 2018 were initially enrolled in this study. The patients were monitored and their clinical, endoscopic and histopathological characteristics were followed up until their final diagnosis was clearly made. Several specimens of intestinal tissue from endoscopic biopsy or surgical excision were collected. One piece was used as paraffin specimen, and the other two pieces were used for MTB culture and Xpert MTB/RIF detection, respectively. Four antigens (38KDa protein, ESAT-6 protein, MPT64, Ag85 complex) of MTB in intestinal tissue were detected by immunohistochemistry. Results: A total of 42 cases of intestinal tuberculosis and 46 cases of CD were included in the experimental analysis. Perianal lesions and longitudinal ulcers were more common in CD patients ($p < 0.05$), while caseous granuloma and annular ulcers were more common in ITB patients ($P < 0.05$). The positive rate of M. tuberculosis detected by Xpert MTB/RIF in intestinal tissue samples of ITB patients was 33.33%, which was significantly higher compared to CD patients ($p < 0.05$) and compared to acid-fast staining smears (9.52%) ($p < 0.05$). The positive MPT64 expression rate in patients with intestinal tuberculosis was 40.48%, which was significantly higher than that observed in CD patients, which was 19.56% ($X2=4.61$, $p<0.05$). Conclusions: The detection of Xpert MTB/RIF in intestinal tissue was conducive to the quick and early diagnosis of intestinal tuberculosis. Xpert MTB/RIF and MPT64 antigen in intestinal tissues had certain value in the differential diagnosis of intestinal tuberculosis and Crohn's disease. The combination of these two methods could improve detection sensitivity.

Background

ITB and CD are chronic intestinal granulomatous diseases that share many similarities in clinical manifestations, imaging, endoscopy and intestinal histopathology. Over recent years, following the population migration, drug-resistant bacterial infection, and the spread of AIDS, the incidence of tuberculosis and ITB have significantly increased [1]. The incidence of CD has also been increasing in China [2]. Although numerous studies have addressed the clinical, pathological and molecular biological detections of the two diseases, the differential diagnosis of ITB and CD still represents a challenging clinical problem, especially in the developing countries with high incidence of tuberculosis where the misdiagnosis and missed diagnosis may lead to serious adverse consequences [3–5].

Xpert MTB/RIF is a new technology that has recently been approved by WHO [6]. It is an *in vitro* diagnostic technique based on semi-nested multiplex real-time fluorescent quantitative PCR using Gene Xpert full-automatic closed nucleic acid detection system. Primers and probes are designed to target the core sequence of rifampicin-resistant determinant regions which are characteristic of *M. tuberculosis.* It can be used to diagnose MTB infection, but also to determine rifampicin resistance (mutation in rpoB sequence).

The whole detection process can be completed in 2 hours. Since its first applications, this technology has been gradually recognized and approved for its diagnostic value in tuberculosis detection, showing high accuracy, sensitivity of 89% and specificity of 98% in the diagnosis of adult pulmonary tuberculosis [7]. Previous studies have investigated the diagnostic value of Xpert MTB/RIF in extrapulmonary tuberculosis [8–10]; nevertheless, researches on the application of Xpert MTB/RIF in the diagnosis of ITB are still very rare.

MTB secrete a variety of antigens with potential diagnostic significance [11–13]. 38KDa protein is a membrane protein, which is closely related to the transport of phosphate. It is an early secretory protein encoded by *Psts* gene that can stimulate T cells and B cells to develop corresponding immune response. ESAT–6 is an early secretory protein of MTB that only exists in MTB and a few pathogenic mycobacteria, but not in non-pathogenic mycobacteria and BCG. MPT64 exists in MTB complex, and most BCG strains cannot secrete this protein. Ag85 complex is an acid transferase of Mycobacterium, where each strain of Mycobacterium can secrete Ag85, and thus has an important role in the late stage of cell wall synthesis of MTB.

In this study, Xpert MTB/RIF detection system was used to detect MTB. The immunohistochemical method was used to detect several tuberculosis antigens in the intestinal tissue samples of ITB and CD patients to clarify the role of Xpert MTB/RIF detection and tuberculosis antigen detection in the differential diagnosis of ITB and CD. Additionally, the clinical, endoscopic and pathological features of CD and ITB were also compared.

Materials And Methods

Patients

Patients who were clinically suspected of having CD and ITB between January 2016 and March 2018 were initially included in this study. Patients who received antituberculosis treatment within three months before the collection of specimens were excluded. The clinical, endoscopic, radiological and biopathological data of the subjects were collected, and the patients were followed up after treatment. Several specimens of intestinal tissue from endoscopic biopsy or surgical excision were collected. One piece was made into paraffin specimen, and the other two pieces were used for MTB culture and Xpert MTB/RIF detection, respectively.

The written informed consent was obtained from all the patients before the clinical trials started and the study was approved by the ethics committee of tongde hospital of zhejiang province.

Diagnostic criteria

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 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.

Xpert MTB/RIF detection of intestinal tissue specimens

Intestinal tissue specimens were decontaminated with 1% NALC NaOH and then were centrifuged. The tissue was mechanically homogenized and suspended with 1 ml of sterile saline solution. About 1 ml of specimen was taken, and 2 times volume of sample treatment solution was added. The solution was mixed by vortex oscillation for 10 seconds, kept at room temperature for 10 minutes, mixed again by vortex oscillation for 10 seconds, and then kept at room temperature for 5 minutes. The mixture of 2 ml was added to Xpert reagent kit, and the kit was put into Gene Xpert instrument for detection. After the reaction, the test results were directly observed under the window of the detection system.

Acid-fast staining and MTB culture of intestinal tissues

Fresh intestinal tissue specimens were decontaminated with 1% NALC NaOH and then were centrifuged. The tissue was mechanically homogenized and suspended in sterile saline solution. The treated samples were examined by acid-fast staining smear, MTB rapid culture and results determination. The specific procedures were carried out in accordance with “The Laboratory Science Procedure of Diagnostic Bacteriology in Tuberculosis”.

Immunohistochemical detection of several tuberculosis antigens in intestinal tissues

Four antigens of MTB (38KDa protein, ESAT–6 protein, MPT64, Ag85 complex) were detected. Four micron thick tissue paraffin embedded slices were taken. Dewaxing, thermal repair and blocking were sequentially performed. Primary antibody was incubated overnight, and secondary antibody was incubated for 1 hour. The slices were washed with PBS, colored with DAB for 5 minutes, and sealed. The procedure strictly followed the instructions of the kit.

Statistical analysis

SPSS20.0 software was used for statistical analyses. Measurement data were expressed as mean ± standard deviation and T-test was used for comparison between groups. Countable data were expressed in rates, and X^2 test or Fisher’s exact probability method were used for comparison between groups. $P < 0.05$ was considered to be statistically significant.

Results

General information

Histological specimens were initially collected from 110 patients, where 22 patients were excluded for diagnosis of non-intestinal tuberculosis or CD. Finally, 42 cases of intestinal tuberculosis (14 cases were surgical specimens) and 46 cases of CD (3 cases were surgical specimens) were included in the experimental analyses.

There were 20 male (47.6%) and 22 female (52.4%) patients with intestinal tuberculosis; the age of onset was 18–59 years old, with an average age of 30.5±11.8 years old. There were 27 male (58.7%) and 19 female (41.3%) patients with CD; the age of onset was 17–61 years old, with an average age of 31.5±12.2 years old. There was no significant difference in gender and age between the two groups.

Clinical features

Clinical features are shown in *Table 1.* The most common symptoms of both CD and ITB were abdominal pain and diarrhea. Abdominal pain was found among 41 patients (89.1%) with CD and 33 patients (78.6%) with ITB; the diarrhea was found in 37 patients (80.4%) with CD patients and 29 (69.0%) patients with ITB. There was no significant difference in abdominal pain and diarrhea among the groups. There were 10 cases (21.7%) among CD patients with perianal lesions, including 6 cases of anal fistula, 3 cases of perianal abscess and 1 case of anal fissure; and there was 1 case among ITB patients with perianal lesion (anal fissure). The observed difference was significant ($P < 0.01$). Longitudinal ulcer was found in 17 patients with CD (36.9%), which was significantly higher than that in ITB patients (6 cases, 14.3%). Annular ulcer was found in 25 patients with ITB (59.5%) which was significantly higher compared to CD patients (7 cases, 15.2%). Among CD patients, 11 (23.9%) had aphthous ulcer, 10 (21.7%) had paving-stone like changes in ulcers and 11 patients (23.9%) had pseudopolyps, which were significantly different from those found in patients with ITB (4 cases, 10.3%; 5 cases, 11.9%; 14 cases, 33.3%); the observed differences were not statistically significant. Caseous granuloma was found in three cases of ITB endoscopic biopsy specimens and 7 cases of ITB surgical specimens, which had definitive diagnostic significance. Non-caseous granulomas were found in 17 patients with ITB (40.5%) and in 12 patients with CD (26.1%); but there was no significant difference between the groups. The difference in lymphocyte aggregation at the bottom of lamina propria between the two groups was also non significant.

Detection and comparison of M. tuberculosis in intestinal tissues of ITB and CD patients

The positive rate of *M. tuberculosis* detected by Xpert MTB/RIF in intestinal tissues of ITB patients was 33.33%, which was significantly higher than that of CD patients ($p < 0.01$). The positive rate detected by Xpert MTB/RIF in 28 biopsy specimens of ITB patients was 14.28% (4/28), and that in 14 surgical specimens was 71.43% (10/14). The positive rate of MTB detected by Xpert MTB/RIF in intestinal tissue specimens of ITB patients (33.33%) was higher than that detected by tissue culture (21.43%), but the difference was not statistically significant. The positive rate of MTB detected by Xpert MTB/RIF in intestinal tissue specimens of ITB patients (33.33%) was significantly higher than that detected by acid-fast staining smear (9.52%). The results are shown in *Table 2.*

Clinical evaluation of detection of M.tuberculosis in intestinal tissues by Xpert MTB/RIF

The specificity of all three methods for detection of *M. tuberculosis* in intestinal tissues was very high. The specificity and positive predictive value of Xpert MTB/RIF for detection of *M. tuberculosis* in intestinal tissues were both 100%. The negative predictive value of Xpert MTB/RIF for detection of *M. tuberculosis* in intestinal tissues (62.2%) was higher than that of tissue culture (58.2%) and acid-fast staining (54.9%), but there was no significant difference. The results are shown in *Table 3.*

Expression of intestinal tuberculosis proteins in patients with intestinal tuberculosis and Crohn's disease

Psts1, MPT64, Ag85B and EAST–6 were expressed in the granular cytoplasm of intestinal granuloma cells in intestinal tuberculosis and Crohn's disease (*Figure 1).*. The positive rate of MPT64 expression was 40.48% in intestinal tuberculosis patients and 19.56% in Crohn's disease patients, and the difference was statistically significant ($X^2 = 4.61$, $P < 0.05$). However, there was no statistically significant difference in the expression of the other three tuberculosis proteins between the two diseases (*Table 4).*.

Detection of M. tuberculosis by intestinal tuberculosis protein combined with Xpert MTB/RIF

The sensitivity and specificity of combined detection of tuberculosis protein MPT64 and Xpert MTB/RIF in the diagnosis of pulmonary tuberculosis were 50.0% and 80.44%, respectively. The sensitivity of combined detection was improved compared to the single detection sensitivity, but the specificity declined.

Discussion

CD and ITB share many similarities in clinical manifestations and signs, which make them difficult to differentiate [14]. Some studies have proposed certain criteria for identifying CD and ITB, but these criteria have been shown to have great limitations [15–17]. In this study, common symptoms of CD and ITB, such as abdominal pain, diarrhea and ascites, had no significant differences between two groups. Perianal lesions in CD patients were significantly higher than in ITB patients, suggesting that perianal lesions had certain value for the differentiation of the two diseases. Perianal lesions are often the first symptom of CD and are closely related to disease activity, which is why clinicians should pay close attention to them. The data in our study showed that the longitudinal ulcer and stenosis in CD patients were significantly higher than that in ITB patients, and the annular ulcer in ITB patients was significantly higher than that in CD patients. These data were consistent with previous reports [18, 19], and they suggest that the longitudinal ulcer and stenosis are the characteristic manifestations of CD, and that the annular ulcer is the characteristic manifestation of ITB. The pathological changes of CD and intestinal tuberculosis usually occur in the submucosa of the intestinal wall. Because of mucosal swelling, the endoscopic biopsy tissues tend to be too small and the sampling too superficial, resulting in low positive rates. In the current study, there were only three cases of caseous granuloma in ITB endoscopic biopsy, while the pathological positive rate was much lower than that of surgical specimens, suggesting that the differential diagnostic value of endoscopic biopsy specimens in the two diseases was much lower than

that of surgical specimens. Non-caseous granuloma was more common in ITB patients than in CD patients, but there was no statistically significant difference. Non-caseous granuloma alone are not enough to completely exclude ITB and should not be considered as a CD-specific manifestation.

At present, WHO recommends Xpert MTB/RIF for rapid diagnosis of pulmonary tuberculosis and some forms of extrapulmonary tuberculosis [20, 21]. Penz *et al.* have conducted a meta-analysis of 36 foreign studies on the application of Xpert MTB/RIF in the diagnosis of extrapulmonary tuberculosis. Their results showed that the overall sensitivity and specificity for extrapulmonary tuberculosis were 77% (95% CI: 66–85) and 97% (95% CI: 94–98), respectively. Subgroup analysis showed significant diagnostic value for the diagnosis of extrapulmonary tuberculosis in different organs. The sensitivity of pleural fluid was low (36%, 95% CI: 26–50), while the meta-sensitivity of lymph node samples was high (87%, 95% CI: 75–95) [22]. A total of 738 clinically suspected extrapulmonary tuberculosis specimens were detected by Xpert MTB/RIF, and the sensitivity and specificity of Xpert MTB/RIF for detecting extrapulmonary tuberculosis were 84.9% and 86.7% respectively. This suggested that Xpert MTB/RIF detection was a promising method for rapid diagnosis of extrapulmonary tuberculosis.However, the detection situation of intestinal mucosa specimens was not specifically shown in this study [23]. Currently, there are few reports on Xpert MTB/RIF detection in intestinal mucosa in China and abroad. In the present study, the biopsy and surgical specimens from 42 cases of intestinal tuberculosis and 46 cases from Crohn's disease were examined by Xpert MTB/RIF. The results showed that the positive rate of *M. tuberculosis* detected by Xpert MTB/RIF in intestinal tissue samples of ITB patients was 33.33%, which was higher than that of CD patients (0%), and the specificity was 100%. This suggested that the detection of Xpert MTB/RIF in intestinal mucosa might have an important role in the differential diagnosis of intestinal tuberculosis and Crohn's disease. In this study, the positive rate of *M. tuberculosis* detected by Xpert MTB/RIF in endoscopic biopsy specimens was much lower compared to surgical specimens, which might be because the endoscopic biopsy specimens were superficial and small. MTB was not evenly distributed in intestinal tissues, and low levels of MTB extracted from mucosal biopsy specimens may lead to false negative results of Xpert MTB/RIF in tissues.

The gold standard for the etiological diagnosis of tuberculosis is *M. tuberculosis* culture. However, the growth of *M. tuberculosis* usually takes 3–8 weeks, and the positive rate of culture is low, which often causes difficulties in diagnosis. Although the routine acid-fast staining method takes only 2–3 hours, this approach has very low sensitivity and specificity of strain identification. Even though it can detect the acid-fast *Mycobacterium,* it cannot distinguish *M. tuberculosis* from other *mycobacterium.* The sensitivity and accuracy of PCR for pathogen detection provides a useful and new method for the diagnosis of *M. tuberculosis* [24–26].

In this study, the detection of *M. tuberculosis* by Xpert MTB/RIF in the intestinal mucosa was compared with traditional methods, and the results showed that the sensitivity of Xpert MTB/RIF was 33.3%, which was significantly higher than that of acid-fast staining (11.9%) and higher than that of bacillus culture (21.4%). The specificity of the three methods was 97.9%, 100% and 100% respectively, without significant difference. The above results indicated that Xpert MTB/RIF had superior sensitivity compared to acid-fast

staining in detection of Mycobacterium tuberculosis, it also had superior detection time compared with *M. tuberculosis* culture, and it had higher specificity. Therefore, the Xpert MTB/RIF was better approach for the early diagnosis and treatment of intestinal tuberculosis.

Over recent years, following the development of immune technology, an increasing attention has been paid to the study of MTB antigen [13, 27]. At present, the main samples used for MTB antigen diagnosis are body fluid and bacterial culture medium. Reports on the application of tuberculosis antigen in ITB tissues are very rare. There are only two reports on 38KDa antigen with limited number of ITB cases (≤ 10 cases). Ihama *et al.* have performed immunohistochemical detections of 38KDa antigen in intestinal paraffin specimens of 10 ITB patients, revealing that 40% of the patients were positive, which in turn suggested that immunohistochemical detection with MTB monoclonal antibody might be an effective and simple diagnostic method for ITB [28]. Ince and colleagues have performed immunohistochemical detection of 38KDa antigen in 45 tuberculosis tissue specimens (including 8 colon tissue specimens, 8 skin specimens, 5 lung tissue specimens and 24 lymph node specimens), and found that 73% of tuberculosis patients were positive, while only 2 out of 28 patients with CD were positive, suggesting that immunohistochemical detection of 38KDa antigen could be used to distinguish the early diagnosis of MTB from CD [29]. Currently, there are no reports on the expression of pstS1 (38KDa) and East–6 antigens in intestinal tissues. The results of this study showed that the expression of Ag85B, pstS1 (38KDa) and East–6 antigen proteins in intestinal tuberculosis and Crohn's disease had no significant difference. The positive expression rates of Ag85B, pstS1 (38KDa) and East–6 antigen in intestinal tissues were 9.52%, 23.8% and 26.2%, respectively. The detection sensitivity of these three proteins in intestinal tissues was low, and could not be improved even by combined detection. Future studies should examine whether other detection methods such as PCR could improve the detection sensitivity.

Jørstad *et al.* have used immunohistochemistry to detect MPT64 antigen in 132 cases of extrapulmonary tuberculosis and non-tuberculosis specimens, and found that compared with the diagnostic composite reference standard, the sensitivity, specificity, positive predictive value and negative predictive value of MPT64 were 69%, 95%, 94%, 75% and 82%, respectively. MPT64 detection had the best performance in TB lymphadenitis patients and children with TB, suggesting that MPT64 detection could be used for routine diagnosis under low resource allocation to improve the diagnosis of extrapulmonary tuberculosis, especially for tuberculous lymphadenitis and children with TB [30]. Purohit *et al.* have studied 51 cases of pulmonary tuberculosis and 38 control specimens of non-pulmonary tuberculosis, including fine-needle aspirations biopsies and formalin fixed biopsy specimens. The sensitivity, specificity, positive predictive value and negative predictive value of anti-MPT64 immunostaining detection were 100%, 97%, 97%, 100% and 82% respectively, suggesting that anti-MPT64 immunostaining detection was a rapid and sensitive method for early specific diagnosis of Mycobacterium tuberculosis infection [31]. This technique can easily be incorporated into routine pathological laboratories. Currently, there are no reports on the expression of MPT64 antigen in intestinal tissues.

In this study, the positive rates of MPT64 antigen in intestinal tuberculosis and Crohn's disease were 40.48% and 19.58%, respectively. The observed difference was statistically significant, which suggested that MPT64 antigen has certain value in the differential diagnosis of intestinal tuberculosis and Crohn's disease. The sensitivity of combined detection with Xpert MTB/RIF in the diagnosis of pulmonary tuberculosis was 50.0%, which was higher compared to the two methods used individually; however, the specificity decreased.

Conclusions

In a word, the detection of Xpert MTB/RIF in intestinal tissue was conducive to the quick and early diagnosis of intestinal tuberculosis. Xpert MTB/RIF and MPT64 antigen in intestinal tissues had certain value in the differential diagnosis of intestinal tuberculosis and Crohn's disease. The combination of these two methods could improve detection sensitivity.

List Of Abbreviations

MTB *Mycobacterium tuberculosis*

ITB intestinal tuberculosis

CD Crohn's disease

Declarations

Ethics approval and consent to participate

The written informed consent was obtained from all the patients before the clinical trials started and the study was approved by the ethics committee of tongde hospital of zhejiang province.

A clear justification in the manuscript has been given to explain that the new procedure was deemed more appropriate than usual clinical practice to meet the patient's clinical need.

Consent for publication

Consent for publication has been obtained from related individual persons whose data were included in the manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors have gained informed consent for publication of the dataset from participants at the point of recruitment to the trial.

Competing interests

The authors declare that they have no competing interests

Funding

This study was supported by public projects of science technology department of zhejiang province (grant number: 2016C33112)

Authors' contributions

Bao-Ying Fei designed research and wrote the paper. Lin Zhou developed the idea for the study. Yu Zhang performed four antigens of MTB detection in intestinal tissue by immunohistochemistry.Yuan-yuan Chen performed Xpert MTB/RIF examination in intestinal tissue . Lin-He Luo collected and analysed the data. . All authors read and approved the final manuscript.

Acknowledgements

This study was supported by public projects of science technology department of zhejiang province (grant number: 2016C33112)

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Tables

Due to technical limitations, the Tables are only available as a download in the supplemental files section.

Figures

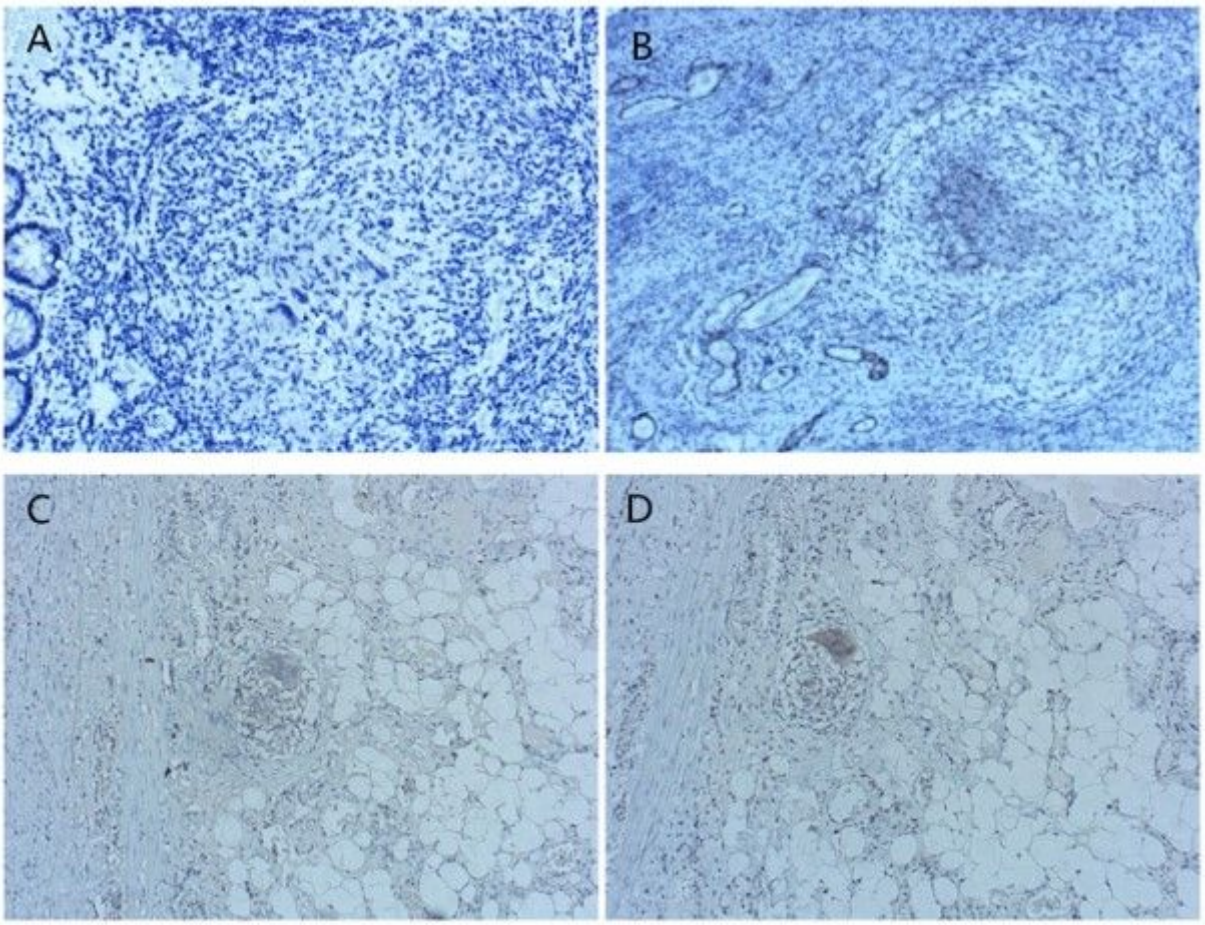

Figure 1

four tuberculosis proteins immunohistochemical view of the colonic specimen of ITB or CD patients (× 200). A: Psts1; B: MPT64;C: Ag85B; D: EAST6

Supplementary Files

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