Full Length Research Paper

Investigation of *Entamoeba histolytica* and *Mycobacterium* spp. in biopsy specimens of patients with inflammatory bowel disease in Turkey

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Intestinal amebiasis and gastrointestinal tuberculosis can mimic inflammatory bowel disease and its exacerbations clinically, pathologically, radiologically and endoscopically. In the existence of IBD and/or either one of these two pathogens, early identification and prompt treatment can improve the clinical course of the patients. The aim of this study was to investigate the presence of *Entamoeba histolytica* and/or *Mycobacterium* spp. in the first diagnostic biopsy specimens of prediagnosed IBD patients in a tertiary education hospital in Ankara, Turkey. As the differentiation of pathologic *Entamoeba histolytica* must be based on isoenzymatic, immunologic or molecular analysis and PCR is a rapid and reliable method for the identification of *Mycobacterium* spp., we investigated the presence of these pathogens in the biopsy specimens of 20 patients who were suspected to have IBD and nine controls, by using PCR-based detection methods. All of them were histopathologically diagnosed as Crohn’s disease and none of the specimens contained these two pathogens. We thought that the low prevalence of both infections in Crohn’s disease patients may have caused our negative findings and loss of pathogens could have lowered the sensitivity. Further studies with larger number of patients are needed to determine the misdiagnosis rate and coexistence of these three diseases.

**Key words:** *Entamoeba histolytica*, *Mycobacterium* spp., inflammatory bowel disease, Crohn’s disease, polymerase chain reaction (PCR), biopsy.

**INTRODUCTION**

Inflammatory bowel disease (IBD) have close resemblance in clinical, pathological, radiological, endoscopic and surgical findings with both intestinal amebiasis and gastrointestinal tuberculosis (GITB), which may lead to diagnostic confusion and challenging treatment. Because of the considerable overlapping features and the difficulty of the differential diagnosis, amebiasis is sometimes falsely diagnosed as inflammatory bowel disease exacerbation (Pai, 2008).

Amebiasis, caused by *Entamoeba histolytica*, is the second leading cause of death from parasitic diseases,
killing 40000-100000 people worldwide annually. (Stanley, 2003). It is also a public health problem in Turkey, especially among people with low socioeconomic status. The infection rate varies according to study methods and regions, however the reported rates dropped significantly since more specific methods than microscopy have been used in the studies (Doganci et al., 1997; Kurt et al., 2008; Tanyuksel et al., 2005). Amebic colitis, usually present as a diarrhoea which may be mild to severe, bloody and with abdominal pain. However, diarrhoea may also be a presenting manifestation of other diseases, including IBD and GITB (Alvares et al., 2005; Espinosa-Cantellano and Martinez-Palomino, 2000; Marcus et al., 2001).

When found together, there can be different relationships between IBD and intestinal amebiasis such as asymptomatic carriage, coexisting infection or superinfection causing exacerbation of colitis. In case of delayed recognition of intestinal amebiasis, treatment of inflammatory bowel diseases with corticosteroids at the presence of E. histolytica trophozoites can lead to serious consequences or an undesirable outcome may occur resulting from delayed antiamebic treatment. Amebic colitis needs to be considered in the differential diagnosis of IBD and its exacerbations. Stool studies, endoscopic biopsy, pathological, immunological and molecular methods are current techniques for the diagnosis of amebiasis.

Tuberculosis is an important public health problem with approximately 9 million new cases reported annually. One percent of the cases is assumed to be abdominal tuberculosis (Sibertiaea et al., 2007). The prevalence is considered to be moderate (20-40 cases /100000) in Turkey (WHO Report, 2009). Ileocecal area is the most frequent site of involvement in gastrointestinal tuberculosis (GITB), which makes it important for differentiating from IBD, especially Crohn’s disease (CD) (Varol et al., 2008). There are also conflicting reports on the possible etiological role of Mycobacterium spp. in the development of inflammatory bowel diseases (Jones et al., 2006; Uzoigwe et al., 2007). Nearly a half of patients with CD from the developing world are initially misdiagnosed and treated as intestinal tuberculosis (Das et al., 2009).

Attempts to detect Mycobacterium tuberculosis in biopsy specimens have met with variable success, with positive PCR noted in 22-75% of intestinal TB patients (Makharia et al., 2009; Pulimood et al., 2008).

Fecal PCR is shown to be useful in detecting intestinal TB and differentiating it from CD (Balamurugan et al., 2006; Balamurugan et al., 2010). However, active pulmonary TB, where swallowed sputum contributes to mycobacterial DNA in the stool, complicates the situation. The sensitivity of fecal PCR increased from 79% to 95% together with the culture of mucosal biopsy specimens.

In this study, our aim was to investigate the presence of E. histolytica and/or Mycobacterium spp. DNA in the initial diagnostic intestinal biopsy specimens of patients who were prediagnosed as IBD.

**METHODS**

In order to investigate Mycobacterium spp., E. histolytica and/or IBD, initial endoscopic biopsy specimens of 20 patients prediagnosed as having IBD and 9 controls who underwent endoscopy with reasons other than IBD, were tested by PCR besides histopathological examination. In each case, biopsy specimens were taken from 1-3 locations of greatest pathologic interest in the colon or small intestine or both. After taken at the gastroenterology clinic, the biopsy specimens were sent to pathology, microbiology and parasitology laboratories. PCR for E. histolytica and Mycobacterium were performed at microbiology and parasitology laboratories. The Institutional Review Board Ethics Committee approved this study (128-3579).

**Extraction of DNA**

DNA was extracted by phenol-chlorophorm extraction method. Briefly, the biopsy specimens were cut into small pieces in a sterile petri dish, and homogenized in 500 µl of sterile distilled water. 4 ml of 0.5 M EDTA buffer, 200 µl 20% SDS and 50 µl Proteinase K were added and incubated at 37°C until a homogenous suspension was obtained (24-48 h, 50 µl of proteinase-K was added at 12th and if necessary at 24th h). The suspension was mixed with an equal volume of phenol-chlorophorm (25:24) and vortexed for 10 min. After centrifugation at 5000 rpm for 10 min at +4°C, the supernatant was transferred to new tube and mixed with an equal volume of chloroform. After vortexing and centrifugation steps, the upper phase was collected and mixed with 1/10 volume of sodium acetate and twice the volume of absolute ice-cold ethanol. The DNA was precipitated by centrifugation at 4000 rpm for 20 min at +4°C, washed once with 70% ethanol and the supernatant was discarded. The pellet was dried and the extracted DNA was suspended in 50 µl of TE buffer (1 M Tris-HCl, 0.5 M EDTA) and stored at -20°C until studied. All centrifugation steps were done at 12,000 rpm in a Beckman-Coulter Microfuge 18 centrifuge.

**E. histolytica real-time PCR**

The presence of E. histolytica DNA was investigated by real-time PCR. The primers and Taqman probes designed on small subunit ribosomal (SSU) RNA gene of E. histolytica (GenBank accession no. X64142) were used (Verweij et al., 2004). The E. histolytica-specific primers consisted of the forward primer (Ehd-239F) 5‘-ATT GTG GTG GTA TAA 3’ and the reverse primer (Ehd-88R) 5‘-GCC GAC GGC TCA TAA CA 3’. The probe used for this assay was VIC-5‘-TCA TGG AAT GAA TTG GCC ATT T-3’ BHQ1. Reaction mixture (20 µl) was prepared by mixing 100 mM KCl, 40 mM Tris-HCl (pH 8.3), 7.5 mM MgCl2, 0.5 µM (each) primer, 0.25 µM probe, 200 µM (each) deoxynucleoside triphosphate, 2.5 U of Taq polymerase and 5 µl DNA. Amplification was performed in 45 cycles under the following conditions: denaturation at 95°C for 15 s, annealing at 55°C for 20 s and extension at 72°C for 20 s in a Bio-Rad DNA Engine Peltier Thermal Cycler, Chromo 4 Real Time PCR Detector (Mexico).

**Mycobacterium spp. PCR**

For amplification of Mycobacterium DNA, 5 µl of DNA was added to the 45 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 10% glycerol, 200 µM (each) dNTP,
that up to two thirds of biopsies of patients with amebic colitis may not show trophozoites in pathological sections (Blumencranz et al., 1983). One of our aims was to investigate if it was possible to diagnose the amebic colitis that was missed in histopathological examination, by using PCR.

The stool microscopy remains the initial approach to diagnosis as a simple and inexpensive method. Nevertheless, this method has a low sensitivity and also leads to false positive results due to microscopically identical Entamoeba spp (Hiiatt et al., 1995). The differentiation of Entamoeba spp. must be based on isoenzymatic, immunologic or molecular analysis. In reference diagnosis laboratories, molecular analysis by PCR based assays is the method of choice for the discrimination between the pathogenic E. histolytica and the nonpathogenic ones. That is why we used PCR which is more specific than microscopic examination of stool.

Enemas administered before endoscopy may wash away the exudate containing the parasite or lyse the trophozoites and may lead to the failure to identify the organisms on biopsy (Juniper, 1978). This issue should also be discussed as a problem when applying PCR from biopsy specimens. Although we examined the whole biopsy specimen, unlike PCR from pathological sections, we were not able to find E. histolytica DNA. Similarly, none of the pathological examination of the patients enrolled in our study revealed E. histolytica.

In a hospital based multicenter study, undertaken to investigate the epidemiologic and clinical characteristics of IBD in Turkey, the incidence was found to be 4.4/100000 and 2.2/100000 for UC and CD respectively (Tozun et al., 2009). They also revealed from the questionnaire, reported from 12 centers throughout the country, that concomitant amebiasis was observed in 17.3% of patients with UC and 1.3% of patients with CD. However, as the method of diagnosis was unclear, the sensitivity or specificity could not be estimated.

Few studies were undertaken about the coexistence of E. histolytica trophozoites and/or cysts in stool samples of IBD patients in Turkey. In one of these studies, 160 IBD patient were explored for the prevalence of amebiasis by using wet mount, concentration and staining methods. The frequency of the parasite was found to be 8.75%, which was significantly higher than that in control patients who were free of gastrointestinal complaints. Therefore they concluded that E. histolytica should be explored in IBD patients (Ustun et al., 2003). In another study from Turkey, amoeba was found in the stool of 8.1% of UC patients on initial diagnosis and in 11.6% of patients during follow up by microscopy. In CD patients these rates were 2.1 and 4.2%, respectively (Ulker et al., 1999). However as the examination based solely on microscopy of specimens, the given rates included all microscopically identical Entamoeba species. Considering that the frequency of E. histolytica is 10% among totally detected microscopically identical...
*Entamoeba* species, the realistic estimation of *E. histolytica* rate in the former study should be lower.

In a recent study from Turkey, among the stool samples of active UC patients which were evaluated for the presence of *E. histolytica* antigen by using ELISA, amoebiasis was detected in 31.5% of the subjects (Ozin et al., 2009). In another study, the prevalence of amoebiasis in 90 UC patients from a high socioeconomic region and 28 UC patients from a low socioeconomic region of Turkey were compared by both microscopy and ELISA. The rate of infection was found to be higher in the second group (53.6%) compared to the first one (32.2%) (Soylu et al., 2009). The results of these two studies were higher than the former studies undertaken in patients who had diarrhoea or IBD in Turkey (Kurt et al., 2008; Ulker et al., 1999; Ustun et al., 2003).

Intestinal tuberculosis should also be suspected during the diagnosis and treatment of IBD particularly in regions where the former disease is endemic (Kirsh et al., 2006). The incidence of TB is 26/100000 according to the Turkish Ministry of Health data published in 2005. The percentage of extrapulmonary TB patients among all TB patients was 28.6% and Gl/peritoneal TB patients consisted 4.5% of all the reported extrapulmonary TB patients (Gümülsuo et al., 2007).

Histologically and microscopically, only 66.5% of GITB cases could be definitely diagnosed (Amarapurkar et al., 2008). It was revealed that 65% of CD has been misdiagnosed as GITB (Tonghua et al., 1981). The values of pathologic examination and PCR from embedded intestinal tissue in differentiating intestinal tuberculosis and CD were compared. It was reported that PCR is more valuable and detected a positivity rate of 64.1% in 39 intestinal tuberculosis specimens. There was no positivity among specimens from patients with CD (Gan et al., 2002). In another study from India where tuberculosis is widely prevalent, the sensitivity of fresh tissue PCR for the diagnosis of intestinal tuberculosis was reported as 82.6% with high specificity (Amarapurkar et al., 2008). *Mycobacterium* spp. DNA besides other viral and bacterial pathogens were sought in the archival tissue of patients with CD and 8.9% of them was found to be positive for *Mycobacterium* spp. (Knösel et al., 2009).

To our knowledge, there are no studies from Turkey exploring the presence of *Mycobacterium* spp. in IBD patients by using PCR. Recently, a study was undertaken in order to evaluate an immunohistochemical staining method in the differentiation of intestinal tuberculosis from Crohn’s disease in biopsy specimens. This method was reported to have sensitivity and specificity of 73 and 93%, respectively (Ozce et al., 2011). Besides, two case reports presenting the misdiagnosis of intestinal tuberculosis as CD were published (Varol et al., 2008; Bar et al., 2005).

In our study, we found neither *E. histolytica* nor *M. tuberculosis* DNA in any of the patient and control specimens. We suppose that, our negative findings may be due to both the low prevalence of these pathogens in patients with CD and the loss of pathogens from the intestinal biopsy specimens. Further studies which will be undertaken by screening higher number of tissue samples would enlighten the rate of misdiagnosis or coexistence of these three diseases.

REFERENCES


