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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, olymerase 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,

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Abbreviations: IBD, Inflammatory bowel disease; **CD,** Crohn's disease; **UC,** ulcerative colitis; **GITB,** gastrointestinal tuberculosis.

killing 40000-100000 people worldwide annually. (Stanley, 2003). It is also a public health problem in Turkey, especiallly among people with low socio-economic 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-Palomo, 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 annualy. One percent of the cases is assumed to be abdominal tuberculosis (Sibertiea 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 homogenious 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 chlorophorm. 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 GTC GTG GCA TCC TAA CTC A- 3' and the reverse primer (Ehd-88R) 5' –GCG GAC GGC TCA TTA TAA CA - 3'. The probe used for this assay was VIC- 5' –TCA TTG 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 $MgCl_2$, 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 30 s and extension at 72°C for 15 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 $MgCl_2$, 10% glycerol, 200 µM (each) dNTP,

0.5 µM (each) primer (Tb11: 5'-ACCAACGATGGTGTGTCCAT and Tb12: 5'- CTTGTCGAACCGCATACCCT), and 1.25 U of *Taq* polymerase (Telenti et al., 1993). After 3 min of first denaturation at 94°C, the reaction was subjected to 45 cycles of amplification (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) which was followed by a 10 min of final extension at 72°C. The amplified 439-bp fragment was subjected to 2% agarose gel electrophoresis and visualized under UV. *Mycobacterium tuberculosis* H37 DNA was used as positive control both alone and mixed with a healthy intestinal tissue. Human TNF-alpha gene was amplified for each patient sample as internal control in order to assess DNA extraction quality, the efficacy of amplification reaction and the presence of inhibitors.

RESULTS AND DISCUSSION

All of the patients were histopathologically diagnosed as having Crohn's disease. We did not detect *E. histolytica* and/or *Mycobacterium* spp. DNA in any of the intestinal biopsy specimens obtained from study and control group patients. Infections due to these two pathogens could not be confirmed in any of the cases.

The colon responds monomorphically to a variety of insults thus making it difficult to differentiate invasive amebic colitis, GITB and IBD. *E. histolytica* is found to be the third frequent patogen causing infection during relapses of IBD being more prevalent in UC patients (Amarapurkar et al., 2008; Mylonaki et al., 2004). Although there have been a number of studies investigating amoeba infection complicating IBD, we have not been able to find a study undertaken using PCR from intestinal biopsy specimens of IBD patients.

The possibility of amoeba infection should always be taken into account in patients with IBD. In patients with a short history of diarrhoea and in countries where amebic infection is endemic, it is important to rule out amebic colitis before commencing steroid therapy for IBD. A latest study carried out in Western Turkey revealed that 59 (2.9%) out of 2,047 stool samples were positive for *E. histolytica/dispar* with microscopy and/or culture (Kurt et al., 2008). Among the positive samples, *E. histolytica* was detected in 14 (23.7%) and 5 (8.5%) samples with PCR and antigen-specific ELISA (EIA), respectively. *E. dispar* was diagnosed in 31 (52.5%) and 52 (88.1%) of 59 samples with species-specific PCR and EIA, respectively. A misdiagnosis of amoebic colitis as an exacerbation of IBD followed by inadvertent treatment with corticosteroids, or the opposite, may be fatal. In a study, 11 patients were described most of which were initially suspected to be tuberculosis or IBD based on the clinical and endoscopic findings. Eight of these patients were diagnosed as amoebic colitis only on histopathological evaluation of the colonic endoscopic biopsy (Pai, 2008). On the other hand, a negative biopsy does not always rule out amebiasis and repeated biopsies may be needed for the diagnosis (Mendonca et al., 1977). Multiple biopsies and PAS stain are sometimes useful in highlighting the cytoplasm of the trophozoites within the exuda and picking up the organism. There is an estimate that up to two thirds of biopsies of patients with amebic colitis may not show trophozoites in pathological sectio ns (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 (Hiatt 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 disccussed 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 amebiasis 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 diarrheoa 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 GI/peritoneal TB patients consisted 4.5% of all the reported extrapulmonary TB patients (Gümü lü 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 (nce et al., 2011). Besides, two case reports presenting the misdiagnosis of intestinal tuberculosis as CD were published (Varol et al., 2008; Ba ar 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.

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