

Investigation of the prevalence of amoebiasis in Izmir province and determination of *Entamoeba* spp. using PCR and enzyme immunoassay

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SUMMARY

Amoebiasis is a common and life-threatening disease. The discrimination of the pathogenic *Entamoeba histolytica* from the non-pathogenic *Entamoeba dispar* could be done by advanced methods such as enzyme immunoassay (EIA) and PCR. The aim of this study was to investigate the prevalence of amoebiasis in Izmir province, and differentiate the *Entamoeba* species by PCR and EIA. Stool samples of 2,047 individuals were examined by direct microscopy, formalin ethyl acetate concentration, trichrome staining and culture, and those found to be positive for *E. histolytica/dispar* by any of these methods were further analyzed by PCR and EIA for species identification. Fifty-nine of 2,047 (2.9%) stool samples were found to be positive for *E. histolytica/dispar* with microscopy and/or culture. Among these 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. Risk factors related to infection with *Entamoeba* spp. and other intestinal parasites included living in shanty houses ($p < 0.01$), a history of recent immigration to Izmir ($p < 0.01$), having no social security ($p < 0.05$) and living with a crowded family ($p < 0.01$). The results demonstrated the significance of amoebiasis as a public health problem among people with low socio-economic status in Izmir province.

KEY WORDS: Turkey, *Entamoeba histolytica*, *Entamoeba dispar*, Prevalence, Epidemiology, Risk factors

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INTRODUCTION

Amoebiasis is a common life-threatening parasitic disease affecting 12% of the world population. It is the third leading cause of mortality due to parasitic infections worldwide, after malaria

and schistosomiasis (Markell *et al.*, 1999). It is estimated that 480 million people are at risk for amoebiasis, predominantly in tropical and subtropical countries, and its mortality rates range between 40,000 and 110,000 annually (Farthing *et al.*, 1996; Markell *et al.*, 1999). The prevalence of *Entamoeba histolytica/dispar* was reported as 4% in the USA, but amoebiasis was the third leading infectious disease and the fourth most common cause of death in autopsy examinations in Mexico, the southern neighbor of the USA, with warmer climate and lower socio-economic and sanitary conditions (Markell *et al.*, 1999).

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Emilie Brumpt was the first to suggest that the differences in symptoms and global distribution of invasive amoebiasis were due to the presence of two morphologically identical species of amoebae: pathogen and non-pathogen. Sargeant and Williams managed to distinguish pathogenic strains of *Entamoeba* complex by isoenzyme typing (Farthing *et al.*, 1996). In 1993, Diamond and Clark used all biochemical, immunological and genetic evidence for the differentiation of *E. histolytica* and *E. dispar*, and redescribed *E. histolytica* (Schaudinn, 1903), formally separating it from *E. dispar* Brumpt, 1925 (Diamond *et al.*, 1993).

The diagnosis of amoebiasis relies primarily on the microscopical examination of stool samples. *E. histolytica* and *E. dispar* are morphologically identical and could not be identified by routine staining methods. Differentiation of *E. histolytica* and *E. dispar* can only be made by isoenzyme analysis and molecular methods (Markell *et al.*, 1999; Tanyuksel *et al.*, 2003) and these methods should be applied to all microscopically-diagnosed cases of amoebiasis to reveal not only the true prevalence of *Entamoeba histolytica* infection, but also the treatment options and public health concerns about amoebiasis (WHO/PAHO/UNESCO, 1997; Huston *et al.*, 1999; Tanyuksel *et al.*, 2003; Tachibana *et al.*, 2000).

In our review on the medical literature, we did not find any comprehensive prevalence study including the identification of *E. histolytica* and *E. dispar* in Turkey. Thus, our aim was to investigate the prevalence of amoebiasis in all counties of Izmir and identify the *Entamoeba* species (*E. histolytica*/*E. dispar*/*E. moshkovskii*) with PCR (polymerase chain reaction) and EIA (enzyme immunoassay) methods.

MATERIALS AND METHODS

Region and scope of the study

Izmir is the third largest city in Turkey, located in the western Anatolia with a population of 3,387,908 individuals inhabiting in a total of 679,200 houses (Population Census Report, 2000). The metropolitan city harbors 9 of the 28 counties and 81% of the whole population, and the populations of the counties range between 13,446 and 782,309 individuals. The study group

was chosen by stratified sampling. The size of the sample in each stratum (county) was calculated in proportion to the population and the house number of the county, with Epi-Info 5.0®. All inhabitants in a house were included in the study. With an estimated average household of 3.58, and the minimum house number necessary to represent the province as 518, a total of 2,072 individuals were found to be necessary to assess the prevalence of intestinal parasites in whole Izmir province. Finally, 2,047 individuals participated in the study, indicating a response rate of 98.8%.

The questionnaires were completed with face-to-face interviews and stool samples of each individual were collected in house visits. The questionnaires contained questions about personal and socio-demographic features such as age, sex, marital status, education and employment, total income and environmental conditions such as social security, frequency of defecation, hand-washing habits, toilet location, source of drinking and non-drinking water, water-keeping conditions, presence of sewage system, number of house inhabitants and house type. Symptoms reported during the interviews were classified in two categories: Gastrointestinal (flatulence, diarrhea, bloating, abdominal cramping, bloody stool, irritable bowel) and extra-intestinal (fatigue, nausea, muscle weakness/pain, headache, fever/night sweats and weight loss).

Parasitological examination

Stool samples were kept at +4°C until they were examined by wet mount, formalin ethyl acetate concentration and trichrome staining, followed by inoculation in Robinson's culture (Garcia *et al.*, 1993; Robinson, 1968). Samples found to be positive for *E. histolytica*/*dispar* in any of these methods were further analyzed by PCR (fresh stool samples) and EIA (samples kept at -20°C until the procedure was performed) for the identification of their species. There were also some unclassified cysts or trophozoite-like bodies detected during the examination of stool samples with microscopy and/or culture, which were classified as suspect stool samples. The frequency of other intestinal parasites was presented in another trial of a large scale project on the prevalence of intestinal parasites in Izmir province (Reg: 04 TIP 018).

TABLE 1 - Comparison of PCR and EIA in the detection of *E. histolytica* in stool samples.

Wet-mount & culture (n= 2,047)	PCR (n=80)		EIA (n=82)	
	<i>E. histolytica</i>	<i>E. dispar</i>	<i>E. histolytica</i>	<i>E. dispar</i>
56 (++) positive)*	12	31	4	52
26 (+)positive)**	2	-	1	-

*Definitely diagnosed cases of *E. histolytica*/*E. dispar* by microscopy and culture. **Suspect cases of *E. histolytica*/*E. dispar* by microscopy and culture.

were detected in 11 of the remaining 13 samples, whereas the other two could not be submitted to PCR. On the other hand, PCR yielded two positive results for *E. histolytica* in 26 suspect cases. Thus, a total of 14 (0.7%, 14/2,047) *E. histolytica* and 31 (1.5%, 31/2,047) *E. dispar* cases were detected with PCR (Table 1).

We conducted the EIA test on a total of 56 cases with definitive diagnosis and 26 suspect cases. The test results indicated 5 (0.2%) stool samples positive for *E. histolytica*: four of them were among the cases with definitive diagnosis while the remaining one was in the suspect group. Fifty-two definitive cases (88.1%) were found to be negative for *E. histolytica* with EIA and considered to be *E. dispar* (Table 1).

On the other hand, 25 suspect cases were found to be negative for *E. histolytica* with EIA; these may be not only *E. dispar* but also other amoeba. Discordant results were also detected between

PCR and EIA. Twelve stool samples were found to be positive by PCR but negative by EIA, and were considered to be positive for *E. dispar*. However, two samples were found to be positive by EIA, but negative by PCR, which were then regarded as *E. dispar*.

The relation between the presence of *Entamoeba* sp. infection and the personal as well as environmental factors was assessed in the study. Among these factors, personal factors such as age, sex, education level, marital status, occupation, monthly income and environmental factors such as eating out, presence of a stable near the house were not found to be significantly correlated to the presence of intestinal parasites.

However, the prevalence of *Entamoeba* sp. and other intestinal parasites was found to be associated with an increasing number of household members (F: 2.487, p<0.01), and higher among people living in shanty houses (χ^2 : 12.275,

TABLE 2 - Statistically significant risk factors for amoebiasis in the study group (n: 2,047).

Characteristics		Parasite-free group (%)	<i>E. histolytica</i> / <i>E. dispar</i> (%)
Location of dwelling	Rural	83.5	1.4
	Urban	75.9	2.4
	Shanty house	73.2	4.0
Duration of living in Izmir	Life long	78.7	2.5
	0-1 year	70.6	5.6
	1-5 years	72.2	1.9
	5-10 years	71.5	4.7
	>10 years	78.3	2.0
Social security	Present	77.0	2.4
	Absent	72.5	4.1
Drinking bottled water	Yes	79.5	1.9
	No*	74.0	3.3

*Drinking tap water and/or artesian water

TABLE 3 - The incidence of symptoms in amoeba-positive and parasite-free individuals.

Symptom	Last 6 months (%)		In the previous week (%)	
	Amoeba (+)	Parasite-free	Amoeba (+)	Parasite-free
Nausea	20.7	15.7	8.6	7.6
Diarrhea	20.7	14.8	10.5	6.0
Abdominal pain	25.9	23.9	10.3	9.8
Loss of appetite	24.1	18.0	12.1	9.1
Weight loss	8.6	11.2	3.4	6.3
Fatigue	27.6	20.4	13.8	10.0
Fever	19.0	12.4	6.9	4.9

$p < 0.01$), new immigrants (χ^2 : 21.535, $p < 0.01$), and among people without any social security (χ^2 : 6.532, $p < 0.05$) (Table 2). On the other hand, the prevalence of the *Entamoeba* spp. and other intestinal parasites was found to be lower among people drinking bottled water than in people drinking tap or artesian water (χ^2 : 8.308, ($p < 0.05$). The clinical symptoms of individuals with amoebiasis both 6 months and one week prior to stool submission are shown in Table 3. None of the symptoms were found to be statistically significant, except that the incidence of weight loss in the previous week were found to be lower in the parasite-free group (χ^2 : 5.74, $p < 0.05$).

DISCUSSION

Amoebiasis is known to be a prevalent infection in tropical and subtropical regions, among crowded populations with poor hygiene and lower socio-economic levels, while it is less common in industrialized countries with higher living standards (Farthing *et al.*, 1996, Markell *et al.*, 1999). *E. histolytica/E. dispar* was detected in 16 of 131 (12.2%) children in a recent study of children with diarrhea in southern Turkey (Koltas *et al.*, 2007). In similar studies conducted in many provinces in eastern and southeastern Anatolia, the prevalence of amoebiasis in the local population ranged between 2.4% and 26.4% (Zeyrek *et al.*, 2006, Tanyuksel *et al.*, 2005, Celiksoz *et al.*, 2005). In addition, it was found to be 8.8% in a

group of Turkish patients suffering from inflammatory bowel disease (Ustun *et al.*, 2003).

The prevalence of *E. histolytica/E. dispar* infection has recently been reported to be 27%, 38.0% and 39.8% in Ecuador, the Philippines and Ghana, respectively (Gatti *et al.*, 2002, Rivera *et al.*, 2006, Verweij *et al.*, 2003). However, it was found to be 8.4% in asymptomatic cyst carriers in Iran and 6% in patients with diarrhea in Nicaragua (Solaymani-Mohammadi *et al.*, 2006, Leiva *et al.*, 2006). In the present study, 59 of 2,047 (2.9%) stool samples were found to be positive for *E. histolytica/E. dispar* after examination with direct microscopy and culture. The lower prevalence of *E. histolytica/E. dispar* infection in Izmir province, located on western Anatolia with a population of over 3 million, was probably due to higher living standards and sanitary levels compared to the provinces in eastern Anatolia.

The basic diagnosis of amoebiasis currently relies on the routine microscopic examination of stool samples with wet-mount, concentration and permanent staining.

However, the presence of neutrophils and macrophages in stool samples may lead to a false diagnosis of amoebiasis during microscopic examination by inexperienced personnel (Acuno-Soto *et al.*, 1993, Haque R *et al.*, 1998). It is also impossible to identify the pathogen *E. histolytica* and non-pathogen *E. dispar* with any microscopic method precluding the determination of the true prevalence of these two species. Today, molecular methods, such as PCR and ribotyping,

antigen detection in stool (EIA) and isoenzyme analysis following culture are used in the identification of these two *Entamoeba* species (Rivera *et al.*, 1998, Markell *et al.*, 1999).

In our study, 59 of 2,047 (2.9%) stool samples were found to be positive for *E. histolytica*/*E. dispar* with microscopy and culture. Among these 59 positive samples, 14 (0.7%) were found to be positive for *E. histolytica*, while 31 (1.5%) were positive for *E. dispar* by species-specific PCR. Many studies have demonstrated that PCR was not only sensitive and specific, but also an effective and relatively simple molecular method for the identification of pathogenic species of *Entamoeba* spp. (Acuno-Soto *et al.*, 1993, Mirelman *et al.*, 1997, Rivera *et al.*, 1998, Huston *et al.*, 1999, Tanyuksel *et al.*, 2003). However, some authors also warned about the susceptibility of PCR to DNA polymerase inhibitors in stool samples which cause false negative results (Tanyuksel *et al.*, 2003, Evangelopoulos *et al.*, 2000). In our study, 11 of 59 previously diagnosed stool samples revealed no band formation during the PCR, which was probably due to DNA polymerase inhibitors in the stool samples.

Another method used in epidemiological studies and the identification of distinct species is the detection of specific antigens in stool samples using monoclonal or polyclonal antibodies (EIA). This method is highly sensitive and specific, and preferred in mass evaluations as it is fast, simple and does not require experienced personnel for evaluation of the results (Haque *et al.*, 2006, Evangelopoulos *et al.*, 2001, Tanyuksel *et al.*, 2003). There were discordant results between the PCR and EIA in some previous studies (Gonin *et al.*, 2003, Leiva *et al.*, 2006, Furrows *et al.*, 2004). Some difficulties were reported with certain samples during the test, which may be due to a number of factors such as the presence of binding substances or inactivating enzymes in the stool samples (Gonin *et al.*, 2003). Here we discovered a similar discordance between the methods: five (0.2%) samples were found to be positive for *E. histolytica* with EIA, and the 52 negative samples were considered *E. dispar*. Twelve samples, initially found to be positive for *E. histolytica* with PCR, were negative with EIA and considered *E. dispar*. In addition, 2 samples found to be positive for *E. histolytica* with EIA were determined as *E. dispar* in PCR. According to the findings both in

the present and earlier studies (Evangelopoulos *et al.*, 2001; Gonin *et al.*, 2003), PCR may be regarded as a more favorable method than EIA, particularly in epidemiological studies on amoebiasis.

Common symptoms of amoebiasis are abdominal pain, watery and sometimes bloody diarrhea, nausea and vomiting. Frequently, pathogenic strains are responsible for symptomatic infections, whereas the non-pathogenic strains cause no concrete symptoms. Asymptomatic cyst carriers are reported for both strains (Farthing *et al.*, 1996, Blessmann *et al.*, 2003). Assessment of the symptoms of the individuals both in the last 6 months and in the previous week revealed no significant relations, indicating that these individuals were probably asymptomatic cyst carriers.

Although the prevalence of amoebiasis found in the present study was relatively low (2.9%), it was obviously elevated with the increasing number of house members, in shanty house districts, recent immigrants, in people without social security and drinking tap or artesian water.

Immigration is a significant factor for the transmission of all sources of infections, including parasites and infections of intestinal parasites are common among both recent and longer term immigrants (Caruana *et al.*, 2006). Being the third biggest province of Turkey with over 3 million inhabitants, Izmir has been a center of attraction for immigrants from eastern Anatolia, in whom our study revealed a higher incidence of amoebiasis. Poor infrastructure in the shanty house districts of immigrants, lower socio-economic status of these individuals, poor hygiene and crowded houses were all found to contribute to the higher prevalence of amoebiasis (Al-Shammari *et al.*, 2001, Gamboa *et al.*, 1998). On the other hand, no significant relations were detected between the prevalence of amoebiasis and age, sex, education level, marital status, employment, family's monthly income, eating out, presence of a stable near the house or the presence of flies.

To our knowledge, there is no comprehensive study on the overall prevalence of *E. histolytica* / *E. dispar* infection in Turkey. Therefore, this study has a unique significance in terms of being the first comprehensive, population-based, local study on amoebiasis, and it is planned to be conducted in more provinces of Turkey in coming years.

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