Differentiation of *Entamoeba histolytica/Entamoeba dispar* by the Polymerase Chain Reaction in Stool Samples of Patients with Gastrointestinal Symptoms in the Sanliurfa Province

Şanlıurfa'da Gastrointestinal Semptomları Olan Hastaların Dışkı Örneklerinde *Entamoeba histolytica/Entamoeba dispar* PCR ile Ayrımı

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ABSTRACT

Objective: We aimed to diagnose amebiasis and also identify *Entamoeba histolytica (E. histolytica)* and *Entamoeba dispar (E. dispar)* in patients with gastrointestinal symptoms in an endemic region in Turkey.
Methods: Stool samples obtained from 181 patients with gastrointestinal symptoms from the Harran University Hospital of Sanliurfa were examined for the diagnosis of amebiasis by the three methods which are as follows:- In house polymerase chain reaction (PCR) targeting the 135 base pair region located on the small-subunit ribosomal RNA (SSU rRNA) gene to differentiate *E. histolytica* from *E. dispar*; and the commercial kit, RIDASCREEN® stool ELISA, that identifies *Entamoeba* sensu lato antigen and microscopical examination of Trichrome stained smears of stool samples.
Results: Positivity for *E. histolytica/E. dispar* complex was found to be 79 (43.6%) by microscopy versus 83 (45.9%) by PCR out of 181 stool samples. A total of 45 patients were found to be positive by the antigen detection method. PCR and microscopy were both positive in 59 samples. The number of patients infected with *E. dispar* (39.8%) was found to be higher than *E. histolytica* (3.3%) while 5 patients (2.8%) had mixed *E. histolytica+E. dispar* infections according to PCR results.
Conclusion: Routine diagnosis of amebiasis by a combination of microscopy and antigen detection technique should be complemented with a PCR assay as a reference test for sensitive differentiation of both species. *(Turkiye Parazitol Derg 2013; 37: 174-8)*

Key Words: *E. histolytica/E. dispar*, PCR, ELISA, microscopy

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ÖZET

Amaç: Çalışmamızda, endemik bir bölgede olan Şanlıurfa'da gastrointestinal semptomları olan hastalarda amebiazisin tanısı ve *Entamoeba histolytica (E. histolytica)* ve *Entamoeba dispar (E. dispar)* tanımlanmasını amaçladık.
Yöntemler: Şanlıurfa'da gastrointestinal semptomu olan 181 hastadan toplanan dışkı örnekleri amebiazis tanısı için aşağıda belirtilen 3 yöntemle incelenmişlerdir: *E. histolytica/E. dispar* ayıran "small-subunit (SSU) rRNA gen" bölgesinde yerleşen 135 bazlık bölgenin hedeflendiği in house PCR, *Entamoeba* sensu lato antijenini gösteren ticari kit RIDASCREEN® stool ELISA ve Trichrome boyama ile mikroskobik inceleme yöntemleri.
Bulgular: Yüz seksen bir dışkı örneğinin 83'ü (%45,9) PCR ile ve 79'u (%43,6) mikroskobi ile *E. histolytica/E. dispar* pozitif bulunmuştur. Kırk beş hasta, antijen saptama yöntemi ile pozitif bulunmuştur. Elli dokuz örnek ise PCR ve mikroskobi birlikte pozitif tespit edilmiştir. *E. dispar* (%39,8) ile enfekte bulunan hastaların sayısı, *E. histolytica* (%3,3) ile enfekte olanlara göre fazla bulunmuştur. Beş hastada (%2,8) ise PCR ile *E. histolytica+E. dispar* mix enfeksiyonu saptanmıştır.
Sonuç: Amebiazisin rutin tanısında mikroskobi ve antijen saptama yöntemlerinin yanı sıra, her iki türün hassas olarak ayırımı için referans test olarak PCR'ın uygulanması önerilmektedir. *(Turkiye Parazitol Derg 2013; 37: 174-8)*

Anahtar Sözcükler: *E. histolytica/E. dispar*, PCR, ELIZA, mikroskopi

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INTRODUCTION

Amebiasis causes up to 100.000 deaths annually all over the world. *E. histolytica*, known as the main agent of intestinal amebiasis causing amebic colitis and liver abscess, is morphologically identical with *E. dispar,* which is accepted as a non-pathogenic commensal parasite (1). Microscopy, stool culture, serological methods including antibody and antigen detection, and molecular tools such as polymerase chain reaction (PCR) are the methods for the diagnosis of amebiasis. Even though microscopy is still the most widespread diagnostic method of amebiasis, it is incapable of distinguishing *E. histolytica* from *E. dispar* and might cause false positive results (2). *In vitro* culturing and isoenzyme analysis of the parasite are not suitable or practical methods for performing routine diagnostic laboratories. More recently, new techniques for identification of the parasite, such as antigen detection by monoclonal antibodies or DNA detection by molecular methods, are used to distinguish the two species in stool samples (3-5). The World Health Organisation (WHO) suggests using accurate differential diagnosis of amoebic species in order to avoid unnecessary treatment applications (only 10% of *Entamoeba* infections really need treatment) (6).

The Sanliurfa province, located in Southeastern Turkey, is on the crossroads between the Mediterranean, Anatolian plateau, and Mesopotamia. The province is situated in a semi-arid plain at 550 m. The average temperature is 18.1°C; the lowest is-12.4°C in February and highest is 46.5°C in August. The average annual relative humidity is 49% and rainfall is 463 mm^3. (Turkish Government Statistical Records, Annual Report-2010). While a number of studies indicated that amebiasis has been endemic in the population, data avaliable about *E. histolytica* and *E. dispar* prevalances in this province are inadequate (7). The aim of the present study was to detect amoebae and identify *E. histolytica* in stool samples of patients with gastrointestinal symptoms by PCR, stool antigen detection kit and microscopy for the standardisation of diagnosis of amebiasis in routine and reference laboratories.

METHODS

Sampling

Stool samples were collected from 181 patients who were admitted to the Harran University Medical Faculty Microbiology Outpatient Clinic Laboratory between June 2005 and June 2006, with the clinical signs of amebiasis and having gastrointestinal symptoms such as diarrhea, bloody and/or mucous stool specimen, abdominal pain, nausea and/or vomiting. None of the patients had a history of travelling. The informed consent forms were collected prior to the sampling and patients who received any medication within the previous three weeks were excluded from the study. Direct smears were prepared soon after collection and stool samples were stored at -80°C. Frozen samples were transferred to the Ege University Medical Faculty Department of Parasitology laboratory on dry ice for performing ELISA and PCR procedures

Microscopy

Direct smears from samples were stained with Trichrome stain and slides were examined three times by different experienced microscopists independently (8).

ELISA

The RIDASCREEN® ELISA (R-Biopharm AG, Darmstadt, Germany, C1701) commercial kit, designed to detect *Entamoeba* sensu lato antigen qualitatively in stool samples, was used for antigen detection in stool samples according to the manufacturer's instructions.

DNA extraction

Genomic DNAs were extracted from 1 gram of stool samples using PSP® Spin Stool DNA Plus Kit (Invisorb® Invitek) according to the manufacturer's instructions. DNA samples were stored at -20°C until PCR was performed.

DNA amplification

Genomic DNA was subjected to PCR using different forward and unique reverse primer sets for *E. histolytica* and *E. dispar* (*E. dispar;* Forward: 5'- TAC AAA GTG GCC AAT TTA TGT AAG TA-3', Reverse: 3'-CTGATCTATCAATCAGTTGGTAGT-5', *E. histolytica* Forward: 5'-GTACAAAATGGCCAATTCATTCAATG-3') which were described previously (3). The target sequence was 135 base pairs (bp) with 35% and 34% Guanin/Cytosine ratios for *E. histolytica* and *E. dispar* respectively in small-subunit (SSU) rRNA gene (9). *E. histolytica* and *E. dispar* positive control DNA's were kindly sent by Dr. Hugues Charest (Canada) and double distilled water was used as the negative control.

Amplification reactions were performed in 50 µL volume with the mix 1X PCR buffer, 1.5 mM $MgCl_2$, 50 µM dNTP, 0.6 mM of primers (Iontek®, Istanbul-Turkey), 2.5U Taq DNA polymerase (Promega®, #M8301) and 10 µL of genomic DNA sample. Amplification consisted of 15 min at 94°C for the first denaturation and 40 cycles of 30 sec at 94°C, 60 sec at an annealing temperature of 51°C and 40 sec at 72°C followed by a final extension of 5 min at 72°C. Aliquots of 10 µL of PCR products were separated in 3% agarose gels (AppliChem®, A2114,0500) using 1xTris-borate-EDTA buffer (TBE) and visualized after staining with ethidium bromide (0.2 µg/mL-1). Amplification reactions for each sample were performed twice in a blinded fashion.

Statistical analysis

Results were analysed according to Analyse it Software (Analyse-it Software Ltd, Leeds, UK). Statistical difference was analyzed using the X2 (chi-square) test. The concordance between the results was determined by using the kappa index measure of agreement. Evaluation of the test results was based on the sensitivity, specificity, positive and negative predictive values, and kappa index agreement. To quantify agreement between assays, PCR was used as the reference test. An A P value less than 0.05 was considered as statistically significant.

RESULTS

Among the 181 patients enrolled in the study, 82 (45.3%) were female and 99 (54.7%) were male, and the mean age of the patients were 25.35±17.2 years. There was no significant correlation between age groups or gender and positivity for *E. histolytica/E. dispar* ($p>0.05$).

Thirteen out of 181 patients were found to be infected with other parasites such as *Giardia intestinalis* (3.3%), *Blastocystis* spp. (1.7%), *Entamoeba coli* (1.1%), *Ascaris lumbricoides* (0.6%) and

Dientamoeba fragilis (0.6%) by microscopy. Among these 13 patients, 8 were coinfected with *E. histolytica/E. dispar*. Comparison of microscopy, ELISA and PCR results were shown in Table 1, 2 and 3. Of the three methods used in the present study, PCR was revealed as having the highest positivity rate with 83 positives (45.9%) for *E. histolytica/E. dispar* complex. The results of microscopy with 79 positives (43.6%) were almost in agreement with PCR. In comparison of the two tests; PCR and microscopy were both positive in 59 samples while 24 samples were positive only by PCR and 20 samples were positive only by microscopy. However, the antigen detection method (RIDASCREEN) revealed much less positivity with only 45 positives.

Differentiation of *E. histolytica* and *E. dispar* in 83 PCR positives was revealed in 6 (7.2%) of the samples to be positive for *E. histolytica* versus 72 (86.7%) positive for *E.dispar*. Five (6.0%) samples were found to be coinfected with *E. histolytica* and *E. dispar* ($p<0.001$). A typical PCR amplification is shown in Figure 1.

A total of 45 patients were found to be positive by the *Entamoeba* sensu lato antigen detection method. Thirty seven out of these 45 cases were also found to be positive by PCR (as *E. histolytica* and/or *E. dispar*). The difference between ELISA and PCR results for *E. histolytica* and/or *E. dispar* was found to be significant ($p=0.0001$). Comparison of both techniques showed that agreement was 69.1% (Kappa=0.36). The sensitivity and specificity of ELISA compared to PCR was 53% and 82.7% respectively (Table 4). Among the 83 samples positive for *E. histolytica* and/or *E. dispar* by PCR, 59 were positive by microscopy. However, 20 samples positive by microscopy were not confirmed with PCR. The difference between microscopy and PCR results for *E. histolytica/E. dispar* was significant ($p=0.0001$). Comparison of both techniques showed 75.7% (Kappa=0.50) agreement. The sensitivity and specificity of microscopy compared to PCR was 71.1% and 79.6% respectively (Table 4).

DISCUSSION

Microscopical diagnosis of *E. histolytica/dispar* complex on stool samples depends on Trichrome staining and requires technical expertise because of the existence of apathogenic forms of amoeba, polymorphonuclear leucocytes (PNL) or artifacts that can be misdiagnosed. Moreover, *E. histolytica* and *E. dispar* cannot be differentiated by microscopy (2, 9, 10). More efficient techniques that allow differentiation must be developed in order to avoid unnecessary treatment when *E. dispar* is present, as recommended by WHO (11).

In Turkey, diagnosis of amebiasis depends on microscopical examination with saline and iodine staining by technicians in most of the diagnostic laboratories. Trichrome staining method is only performed in certain specialised parasitology laboratories.

The incidence of *E. histolytica/E. dispar* by microscopy was found to be 0-17% and 2.5-13% in Turkey and in the Sanliurfa province, respectively, and differentiation between *E. histolytica* and *E. dispar* with antigen detection methods was performed in only a few studies. TechLab *E. histolytica* II EIA was performed in 380 stool samples from patients with gastrointestinal symptoms in two endemic cities of Eastern and Southeastern Turkey, and 14 (15.4%) out of 91 microscopically positive specimens were found to be positive for *E. histolytica* (7). In another study; out of 87 suspected stool specimens from Sanliurfa, 19 (21.7%) and 23 (26.4%) were positive for *E. histolytica/E. dispar* by ELISA (Ridascreen® Entamoeba; R-Biopharm AG, Darmstadt, Germany) and microscopy respectively (12).

Table 1. Comparison of Trichrome staining, PCR and ELISA results

Methods	Negative (%)	Positive (%)
Trichrome staining	102 (56.4)	79 (43.6)
PCR	98 (54.1)	83 (45.9)
E. histolytica	175 (96.7)	6 (3.3)
E. histolytica+ E. dispar	176 (97.2)	5 (2.8)
E. dispar	104 (57.4)	72 (39.8)
Ridascreen	136 (75.1)	45 (24.9)

Table 2. Comparison of PCR and antigen detection method results

PCR	Ag negative	Ag positive
E. histolytica	4	2
E. dispar	39	33
E. histolytica+E. dispar	3	2
Negative	90	8
Ag: Antigen		

Table 3. Comparison of PCR and Trichrome staining results

		Trichrome		
		Positive	Negative	Total
PCR	Positive	59	24	83
	Negative	20	78	98
	Total	79	102	181

There have been many publications on amebiasis epidemiology in the past without differentiation of *E. histolytica/E. dispar* and the incidence was reported between 10% and 80% in different localities of the world in the 1990s (13). PCR-RFLP was used successfully for the first time in the differentiation of *E. histolytica/E. dispar* in 1991 (14) and then many studies were carried out nreporting that PCR was a specific and sensitive diagnostic method as well as a valuable tool for the molecular epidemiological studies (9, 12, 15-19). PCR has also some disadvantages, such as the presence of PCR inhibitors and DNA damaging substances in the stool (5).

In one study, ninety-five stool samples from 84 patients have showed 68, 63 and 55 *E.histolytica* or *E. dispar* positives by PCR, microscopy and ELISA respectively. PCR and ELISA showed 85% concordance and PCR was found to be more sensitive and specific than ELISA (9). In another study, microscopically positive 207 samples were differentiated as 5.7% *E. histolytica* and 94.3% *E. dispar* by PCR (10). Among 5378 travelers, 103 laboratory-confirmed amebiasis cases were detected. The results of various diagnostic tests were compared and stool microscopy and ELISA

Table 4. Evaluation of results of Trichrome staining and ELISA methods according to PCR

	Sensitivity (%)	Specifity (%)	PV (%)	NV (%)	Agreement (%)	Kappa index (%)
Trichrome	71.1 (56.9-85.2)	79.6 (66.0-93.1)	74.7 (60.3-89.0)	76.5 (63.0-89.9)	75.7 (64.0-87.3)	50.9
ELISA	53.0 (38.8-67.1)	82.7 (69.0-96.2)	72.1 (56.8-87.0)	67.5 (54.5-80.0)	69.1 (57.4-80.0)	36.4

The values in parentheses represent 95% Confidence Interval
PV: Positive predictive value NV: Negative predictive value

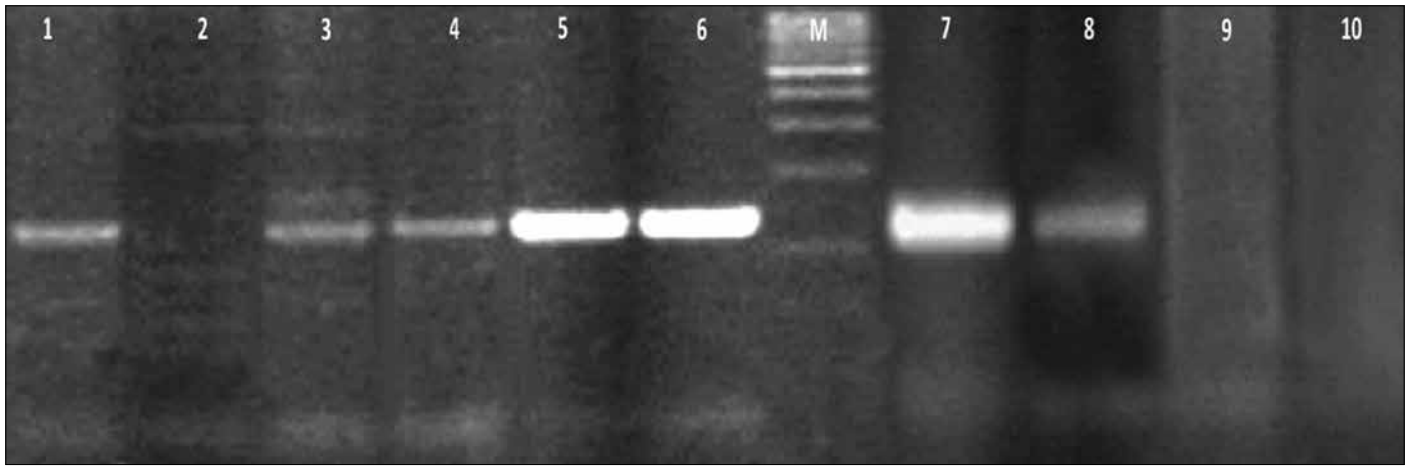

Figure 1. Sample amplification of stools containing or not containing *E. histolytica* and *E. dispar*. Lanes 1 and 6, *E. dispar* amplification; M: marker; lanes 7 to 10, *E. histolytica* amplification; lanes 1, 3, 4, 5 and 8, positive samples; lanes 6 and 7 positive control.

were found to be positive with 82.5 and 93.9%, respectively. Positive samples detected by screening tests were also subjected to PCR with the detection of 9.7% and 88.3% of *E. histolytica* and *E. dispar* respectively (20). In contrast to many studies, Stark et al. (21) have reported that ELISA kits were 1000 to 10,000 times less sensitive than PCR and not useful for the detection of *E. histolytica* in stool samples from patients in non endemic geographical regions. We also have similar results in the present study. One hundred eighty one patients have showed 83, 79 and 45 *E. histolytica and/*or *E. dispar* positives by PCR, microscopy and ELISA respectively. Six (7.2%) *E. histolytica*, 72 (86.7%) *E. dispar* and 5 (6%) *E. histolytica*+*E. dispar* co-infections were detected out of 83 PCR positives and 24 of them were found to be negative by microscopy. Overall, the concordance of PCR and ELISA was found to be 69.1%. The specificity and positive predictive values of ELISA compared to PCR were 82.7% and 72.1% respectively. In accordance with other studies, our results (87% *E. dispar* infections) confirm that *E. dispar* is about ten times more prevalent in fecal samples than *E. histolytica* (20, 21).

A high prevalence of *E. moshkovskii* infection (21.1%) has been detected in preschool children in Bangladesh (22). In another study, *E. moshkovskii* (1.1%) was shown as a rare human parasitic infectious agent in asymptomatic cyst passers in Iran, a neighbouring country of Turkey (23). Two *E. moshkovskii* cases were also reported in Turkey (24). In the present study, 20 samples were found to be negative by PCR out of 79 microscopically positives. These 20 negatives can be explained by either having *E. moshkovskii* or presence of inhibition factors for PCR assay in stool samples. In ouropinion, there is a need for identification of *E. moshkovskii* in stool samples, especially for PCR negative and microscopically positive samples.

CONCLUSION

Accurate differentiation of invasive *E. histolytica* from the morphologically identical commensal *E. dispar* is crucial for clinical management of patients and epidemiological investigation of amebiasis. Although this study has been providing comparable results of microscopy, ELISA and PCR, none of these methods can detect all positives alone. According to our results, microscopy is a simple analysis, but it is subjective, needs experience to evaluate and should be combined with complimentary methods such as antigen detection and PCR for identification of the species to avoid false and/or insufficient diagnosis and treatment applications.

Conflict of Interest
No conflict of interest was declared by the authors.

Peer-review: Externally peer-reviewed.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Author Contributions
Concept- F.Y.Z., N.T.; Design - F.Y.Z., N.T., S.T.; Supervision - S.T., U.A.; Funding - N.T., S.T.; Materials - F.Y.Z., A.U.; Data Collection and/or Processing - F.Y.Z., N.T., A.U.; Analysis and/or Interpretation - F.Y.Z., S.T., N.T.; Literature Review - F.Y.Z., A.U., S.U.; Writer - F.Y.Z., N.T., S.T.; Critical Review - F.Y.Z., U.A., S.T., N.T., A.U., S.U.; Other - S.T., N.T.

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Hasta Onamı: Yazılı hasta onamı bu çalışmaya katılan hastalardan alınmıştır.

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