

An investigation of the relationship between clinical features of amoebiasis and *Entamoeba histolytica* genotypes*

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Aim: To determine the presence of *Entamoeba histolytica*/*E. dispar* and *E. moshkovskii* in stool samples, tRNA-based short tandem repeat gene polymorphism in *E. histolytica* isolates, and the relationship between amoeba load and clinical outcome in studies.

Materials and methods: This study involved 840 stools samples of individuals having diarrhea/dysentery and individuals who were asymptomatic by using microscopy, culture, *E. histolytica* antigen ELISA, and conventional/real-time PCR methods.

Results: Of the 840 samples analyzed, 4.3% (36/840), 2.6% (22/840), and 7.4% (62/840) of the stool samples were determined to be positive by *E. histolytica* antigen ELISA, and real-time PCR for *E. histolytica* and *E. dispar*, respectively. Thirty-five of the 62 (56.4%) samples positive for *E. dispar* and 20 of the 22 (91.0%) samples positive for *E. histolytica* were from dysenteric individuals as revealed by real-time PCR. Although there was no statistically significant difference in patients with diarrhea, a correlation might be seen between amoeba load and clinical outcome in those infected with *E. histolytica*, since amoeba load was usually determined 10³ copies/mL or higher in patients with diarrhea. In this study, 3 different genotypes were defined in 16 isolates by using 6 loci (A-L, N-K2, D-A, R-R, S-D, and S^{TGA}-Q).

Conclusion: Our results demonstrated that real-time PCR is a useful, reliable, and sensitive method for the determination of *E. histolytica* in stools and for differentiation from *E. dispar*. It is suggested that parasite load might affect clinical outcome.

Key words: *Entamoeba histolytica*, amoebiasis, real-time PCR, tRNA gene, genotyping

Introduction

Amoebiasis is an important protozoan infection ranking third among parasitic diseases on the basis of death rate after malaria and schistosomiasis. There are still problems concerning the determination of *E. histolytica*, the causative agent of amoebiasis. Nine of each 10 patients have a high probability of having *E. dispar* for amoebiasis in cases reported to be positive for *E. histolytica*. Therefore, patients might have been misdiagnosed and overtreated (1).

Diagnosis of amoebiasis using molecular methods is useful not only in terms of diagnosis and but also for epidemiological studies through removing possible microscopy mistakes (2–6). Currently, *E. histolytica* antigen-specific ELISA or DNA determination (real-time PCR is more sensitive) tests are considered to be the most scientific alternatives for definitive diagnosis (7–9). The fact that *E. histolytica* presents a clinically different picture in different geographical places causes problems in the diagnosis of infection. There

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are several studies indicating genetic differences of *E. histolytica* (10,11). It was also shown by genotyping that there are short repetitive structures (short tandem repeat (STR)) inside intergenic regions between tRNA genes of different *E. histolytica* strains (12,13).

There were 3 goals in this study: i) to obtain information about the actual prevalence of amoebiasis by the classical inspection method, *E. histolytica* antigen ELISA, and *E. histolytica* specific conventional/real-time PCR for stool samples, collected from asymptomatic individuals as well as from patients with diarrhea/dysentery in Diyarbakır, a region considered to be endemic; ii) to determine amoeba load for *E. histolytica* and *E. dispar* positive patient samples by real-time PCR and to investigate the relationship between amoeba load and clinical outcome; and iii) to genotype via tRNA gene study of *E. histolytica* positive isolates.

Materials and methods

The procedures described below were applied to stool samples collected from individuals suffering from diarrhea/dysentery and individuals who were asymptomatic in the laboratories of the Department of Medical Microbiology, Dicle University.

Stool samples were recorded, and bacteriological culture, parasitological examination (ova and parasite (O&P)) (0.85% NaCl and Lugol's iodine examination, trichrome staining, acid-fast staining, flotation method) (14), parasitological culture (Robinson medium), and antigen ELISA (specific *E. histolytica* antigen-ELISA (TechLab *E. histolytica*, USA)) (15) were used.

A commercial DNA extraction kit (Qiagen, QIAamp DNA stool mini kit, Valencia, CA, USA) was used for the DNA extraction as described by the manufacturer (15).

The following tests using extracted DNA samples were performed:

- A. *E. histolytica* and *E. dispar* were determined using real-time PCR (4,16),
- B. *E. histolytica* and *E. dispar* were determined using conventional PCR (17),
- C. *E. moshkovskii* was detected by nested PCR (18),
- D. tRNA-STR locus PCR experiments (12).

Statistical analysis

Chi-squared and kappa coefficients were used for statistical analysis using SPSS 11.5 for Windows (Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

Results

Results obtained from the analysis of stool samples collected from a total of 840 individuals suffering from diarrhea/dysentery and asymptomatic (no diarrhea or dysentery) using microscopic O&P examination, culture, *E. histolytica* antigen ELISA, and molecular tests are summarized in Table 1.

The DNA samples were tested for the presence of *E. histolytica* and *E. dispar* by real-time PCR in the same tube. To determine the analytical sensitivity of real-time PCR, *E. histolytica* (ATCC 30190) and *E. dispar* (ATCC 50631) strains were diluted 10-fold. While the sensitivity of real-time PCR was 10^2 copies/mL, that of conventional PCR was 10^3 copies/mL. Twenty-two samples were determined as *E. histolytica* positive and 32 samples as *E. dispar* positive by real-time PCR. *E. moshkovskii* was not observed in the samples using the conventional nested PCR analysis.

For studies of stool samples via different methods, the ratio of *Entamoeba* spp. trophozoite/cyst by microscopic method was not statistically significant for dysenteric and asymptomatic individuals ($P = 0.811$). Accordingly, while there was no statistical significance between the 2 groups for the presence of *E. histolytica* according to real-time PCR ($P = 0.083$), there were significant differences between the 2 groups ($P = 0.002$ and $P < 0.001$, respectively) for *E. histolytica* antigen ELISA and *E. dispar* real-time PCR (Table 2).

For all of the samples ($n = 840$), there was a significant difference ($P < 0.001$) between microscopy and stool samples for the *E. histolytica* antigen ELISA positivity frequency. There was no statistical relationship ($P = 1.00$) between determination of *E. histolytica* positive samples ($n = 22$) by real-time PCR and ELISA positivity but there was a significant difference ($P = 0.002$) between microscopic and ELISA positivity for *E. histolytica* determined samples by real-time PCR and for *E. dispar* negative samples ($n = 756$) (Table 3).

Table 1. Results of samples collected from the study group.

Total number of samples	840
Symptom existence	
Diarrhea	631
No diarrhea/dysentery problem	209
Intestinal parasite presence	129*
Stool microscopy	
Erythrocyte presence	48
Leukocyte presence	41
Erythrocyte and leukocyte presence	58
<i>Entamoeba</i> spp. presence	84**
Results of test and cultures	
Positivity of <i>E. histolytica</i> antigen ELISA	36
Growth in Robinson medium	21***
Positivity of bacterial culture	2
Molecular methods	
Positivity of <i>E. histolytica</i> real-time PCR	22
Positivity of <i>E. dispar</i> real-time PCR	62

* *Giardia intestinalis* trophozoite/cyst in 44 patients, *Blastocystis hominis* trophozoite in 40 patients, *Cyclospora cayetanensis* oocyst in 7 patients, *Entamoeba coli* cyst in 5 patients, *Chilomastix mesnili* cyst in 4 patients, *Hymenolepis nana* egg in 3 patients, *Iodamoeba bütschlii* in 2 patients, 1 had *Enterobius vermicularis*; 20 patients had 2 parasites simultaneously (3 had *G. intestinalis* + *E. coli*; 6 had *G. intestinalis* + *B. hominis* trophozoite; 5 had *B. hominis* trophozoite + *E. coli* cyst; 1 had *G. intestinalis* trophozoite/cyst + *C. cayetanensis* oocyst; 1 had *C. cayetanensis* oocyst + *B. hominis* trophozoite; 1 had *C. cayetanensis* oocyst and *E. coli* cyst; 2 had *H. nana* + *E. coli* cyst; 1 had *Entamoeba hartmanni* cyst and *B. hominis* trophozoite), 3 patients had 3 parasites at the same time (*Hymenolepis nana* egg + *B. hominis* trophozoite + *I. bütschlii*; *B. hominis* trophozoite + *E. coli* cyst + *C. mesnili* cyst; and *B. hominis* trophozoite + *I. bütschlii* cyst + *C. mesnili* cyst).

** *Entamoeba* spp. trophozoite in only 13 of 84 patients, *Entamoeba* spp. cyst in 69 of 84 patients, and *Entamoeba* spp. trophozoite in 2 of 84 patients were determined as a result of microscopic examination of stool samples.

*** Stool samples were cultivated in Robinson medium for culture but there was no growth, but combination with bacterial, fungal and/or other parasites was observed. *E. histolytica* was not determined (denoted as negative) when experiments were performed for DNA isolates obtained from microscopy as well as culture by conventional and real-time PCR. *E. histolytica* could not be diagnosed in the xenic Robinson medium. Therefore, the results of culture were excluded from the study due to lack of optimization and tendency for causing misidentification.

Table 2. Comparison of clinical (diarrhea/asymptomatic) and microscopy (*Entamoeba* spp. trophozoite/cyst), antigen ELISA test for stool samples, and *E. histolytica* real-time PCR results for stools samples (n = 840).

	Microscopy (<i>Entamoeba</i> spp. trophozoite/cyst)		<i>E. histolytica</i> antigen ELISA for stools		<i>E. histolytica</i> real-time PCR		<i>E. dispar</i> real-time PCR	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Diarrhea (n = 631)	64	567	19	612	20	611	35	596
Asymptomatic (n = 209)	20	189	17	192	2	207	27	182
P	0.811		0.002		0.083		<0.001	

Table 3. Comparison of real-time PCR, microscopy, and ELISA results of stools samples.

Sample groups	Test results and percentage (%)	
	Microscopy (<i>Entamoeba</i> spp. trophozoite/cyst) positivity	Stools <i>E. histolytica</i> antigen ELISA positivity
All samples (n = 840)	84 (10.0%)	36 (4.3%) P < 0.001*
<i>E. histolytica</i> real-time PCR positive samples (n = 22)	4 (18.1%)	4 (18.1%) P = 1.00**
<i>E. histolytica</i> and <i>E. dispar</i> real-time PCR negative samples (n = 756)	57 (7.5%)	29 (3.8%) P = 0.002*

*Significant difference.

**No significant difference.

Additionally, among *E. dispar* real-time PCR positive samples (n = 62), 21 samples (33.8%) were found to be positive by microscopy (*Entamoeba* spp. trophozoite/cyst) and 3 were positive (4.8%) by fecal *E. histolytica* antigen ELISA.

The frequency of erythrocyte and/or leukocyte existence in samples in which *Entamoeba* spp. trophozoite/cyst was observed by microscopy was quite high (46.4%). In addition, erythrocytes were observed in only 1 sample that was *E. histolytica* positive by real-time PCR, leukocytes were detected in 1 sample, and erythrocytes together with leukocytes were observed in 2 samples. No

erythrocytes or leukocytes were observed by microscopy in 18 samples. There were significant differences and medium level coherence (P < 0.001, kappa = 0.241) between observation of *Entamoeba* spp. trophozoite/cyst by microscopy and erythrocyte existence frequency by microscopy.

There was a significant difference and poor coherence (P < 0.001; kappa = 0.109) between the results of *E. histolytica* antigen ELISA and those of *E. histolytica* real-time PCR. Real-time PCR results were within the reference range. For *E. histolytica* antigen ELISA, the sensitivity, specificity, positive predictive value, negative predictive value, and test

general accuracy were calculated as 18.1%, 96.1%, 11.1%, 97.7%, and 94.0%, respectively. There was no significant difference and poor coherence ($P = 0.195$; kappa = 0.035) between the results of sample-specific *E. histolytica* by microscopy and real-time PCR. When real-time PCR results were taken as reference, the microscopy sensitivity, specificity, positive predictive value, negative predictive value, and general accuracy were calculated as 97.6%, 88.3%, 18.1%, 87.7%, and 4.7%, respectively. There was no significant difference and poor coherence ($P = 0.79$; kappa = 0.060) between the results of stool sample *E. histolytica* by microscopy and ELISA.

Although amoeba load was determined as 10^3 copies/mL both in diarrheal patients and asymptomatic individuals who were diagnosed with *E. histolytica* real-time PCR, the difference was not found to be statistically significant ($P = 0.089$) between these groups.

Moreover, there was no significant difference ($P = 0.064$) in copy number for *E. dispar* real-time PCR positive cases ($n = 62$) between diarrhea patients and asymptomatic individuals ($P = 0.064$) (Table 4).

E. histolytica-specific primer pairs with tRNA-based polymorphic STR locus gene directed nested PCR method was applied (Figure 1) and 3 different genotypes were observed in the isolate. The genotypes were named DU-1, DU-2, and DU-3 (Table 5).

1. Fourteen of 16 *E. histolytica* positive tRNA-based genotyped patients had diarrhea.
2. Three different genotypes were observed during tRNA-based genotyping. The most frequently observed genotype was DU-1 among them (9/16). The least frequently observed genotype was DU-2 (1/16).
3. For DU-1, 1 of the 9 patients was asymptomatic and the rest had diarrhea. Seven of the diarrhea patients had 10^2 parasite load, whereas the remaining 1 had 10^3 parasite load. The asymptomatic patient had 10^3 parasite load. DU-2 genotype was only found in 1 diarrheal patient and the parasite load was observed to be 10^2 . One of the 6 patients with DU-3 was asymptomatic and the remaining 5 patients had diarrhea. Four of the diarrhea patients had 10^2 parasite load but only 1 had 10^3 parasite load. The asymptomatic one had 10^2 parasite load.

Table 4. Real-time PCR *E. histolytica*/*E. dispar* positive cases copies/numbers according to clinical outcome.

	Clinical outcome	Copies/mL	Case number
Positive real-time PCR <i>E. histolytica</i> (n = 22)	Diarrhea (n = 20)	10^2	2
		10^3	17
		10^4	1
	Asymptomatic (n = 2)	10^2	1
		10^3	1
Positive real-time PCR <i>E. dispar</i> (n = 62)	Diarrhea (n = 35)	10^2	7
		10^3	11
		10^4	11
		10^5	3
		10^6	3
	Asymptomatic (n = 27)	10^2	6
		10^3	6
		10^4	7
		10^5	5
		10^6	3

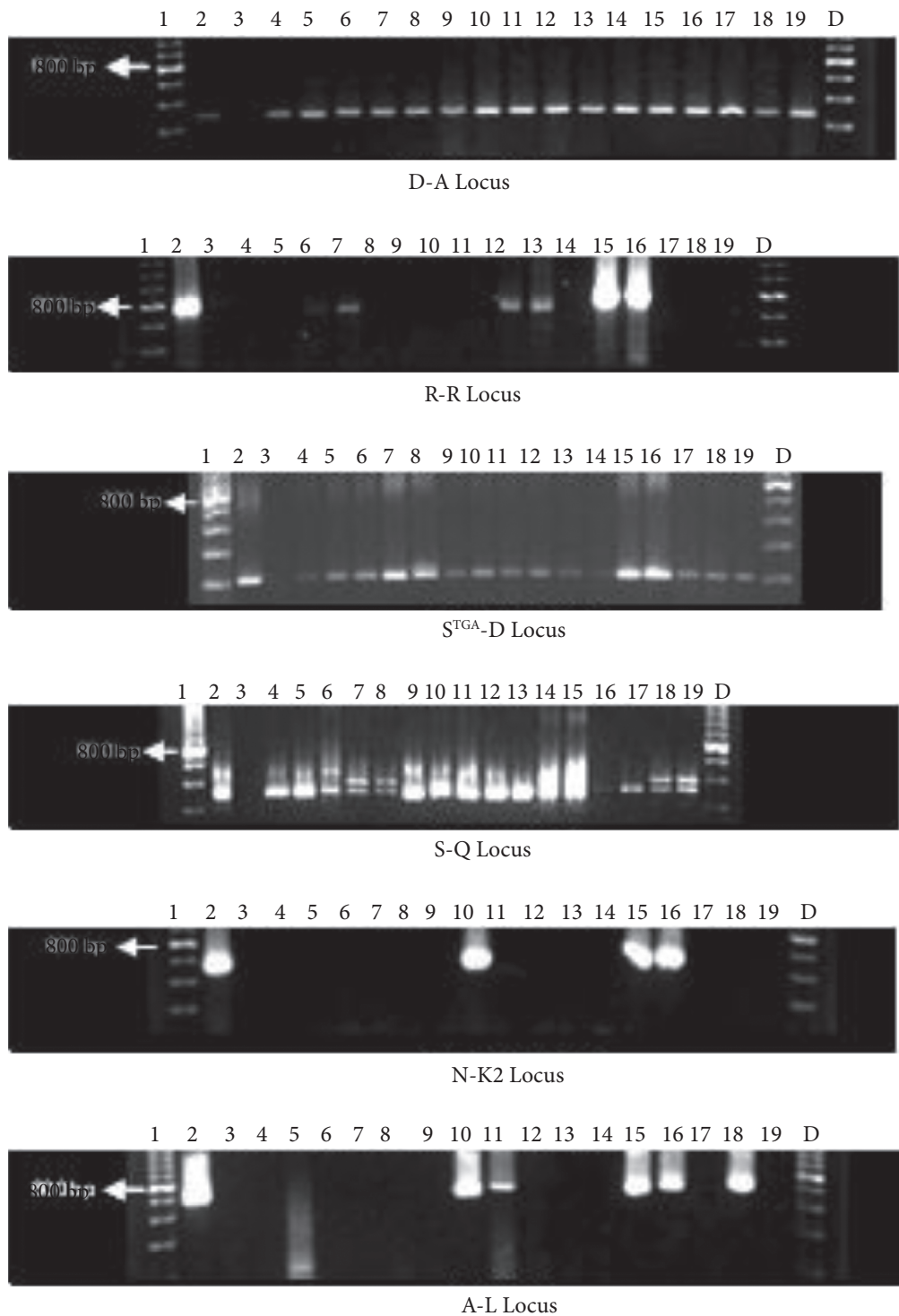


Figure 1. Fragment length polymorphisms in 6 loci from *E. histolytica* isolates, using tRNA-linked STR array method. Line 1: 100 bp DNA marker. Lines 2 and 3: Reference isolate (HM-1:IMSS) and negative control. Lines 4-19: *E. histolytica* positive isolate (7, 53, 115, 33, 77, 83, 84, 104, 132, 141, 284, 313, 317, 324, 349, and 606 numbered isolates, respectively).

Table 5. tRNA-based genotyping of positive *E. histolytica* isolates from clinical samples at 6 loci.

Isolate no.	Clinical outcome	Parasite load (copies/mL)	D-A	A-I	R-R	N-K2	S ^{16A} -D	S-Q	Genotype
7	diarrhea	10 ²	A	-	-	-	A	A	DU-1
33	diarrhea	10 ²	A	A	A	A	A	F2	DU-3
53	diarrhea	10 ²	A	-	-	-	A	A	DU-1
77	diarrhea	10 ²	A	A	-	-	A	F2	DU-3
83	diarrhea	10 ²	A	-	-	-	A	A	DU-1
84	diarrhea	10 ²	A	-	-	-	A	A	DU-1
104	diarrhea	10 ²	A	-	-	-	A	A	DU-1
115	diarrhea	10 ²	A	-	-	-	A	F1	DU-2
132	diarrhea	10 ²	A	A	A	A	A	A	DU-1
141	diarrhea	10 ²	A	A	A	A	A	A	DU-1
284	diarrhea	10 ³	A	-	-	-	A	F2	DU-3
313	diarrhea	10 ²	A	A	A	-	A	F2	DU-3
317	diarrhea	10 ³	A	-	A	-	A	A	DU-1
324	asympt.	10 ³	A	-	-	-	A	A	DU-1
349	asympt.	10 ²	A	-	-	-	A	F2	DU-3
606	diarrhea	10 ²	A	-	-	-	A	F2	DU-3

A: same band; F1: different 1; F2: different 2; (-): no band for PCR

Discussion

In summary of the results for diagnosis in the study:

- After examination of stool samples, 84 *Entamoeba* spp. trophozoites/cysts were observed in 840 samples. *E. histolytica* was observed by real-time PCR in only 4 of them and *E. dispar* was observed in 21 of them. *E. dispar* was observed 5 times more frequently than *E. histolytica*. Microscopic examination (O&P) was not highly sensitive and specific in every case and so it can easily lead to misdiagnosis (especially fecal leukocyte). This result is in accordance with previous studies (19,20).
- The frequency of erythrocyte and/or leukocyte existence in *Entamoeba* spp. trophozoite/cyst observed samples by microscopy was quite high (46%). However, the erythrocytes and/or

leukocytes were observed in only 4 (18%) of the *E. histolytica* positive samples by real-time PCR.

- Separately, in 129 patients 1, 2, or 3 intestinal parasites were observed at the same time. Eighty-four patients had typical intestinal parasites such as *G. intestinalis* and *B. hominis*. This revealed that not only *E. histolytica* but also other parasites should be taken into consideration in diarrhea cases.
- A significant difference and poor coherence were obtained for samples in terms of *E. histolytica* antigen ELISA and specific *E. histolytica* real-time PCR results. ELISA shows weakness in comparison with microscopy for amoebiasis diagnosis. This result was consistent with other studies in the literature (21,22). The differentiation of *E. histolytica* from *E. dispar* is crucially important

for the definitive diagnosis of amoebiasis, and the real-time PCR is a reliable method for this.

- e. As a result, while microscopy and stool antigen ELISA have low sensitivity (18% for both methods), ELISA has higher specificity (96.1%) than microscopy has (87.7%) with respect to the real-time PCR method. In particular, the low sensitivity of ELISA is conspicuous and in accordance with the study performed by Stark et al. (23).

A precise diagnosis requires that the same reaction conditions are used for standardization. Real-time PCR is an attractive technique for laboratory diagnosis of infectious diseases because of its characteristics that eradicate post-PCR analysis, leading to shorter turnaround times, with a decrease in contamination of laboratory environments and reduced reagent costs (24). In a report about a travel clinic, microscopy and antigen ELISA served as initial screening tests, and stool samples with negative results in these tests were not subjected to PCR analysis or to serological testing. Therefore, a number of patients with *Entamoeba* spp. infections may not have been detected as such (25). In patients in whom only 1 sample was collected, the clinical course and probable complications were not monitored and the laboratory results (*E. histolytica*/*E. dispar* existence and tRNA-based genotype determination) were compared with the presented clinical pictures (diarrhea/dysentery/asymptomatic) only as done in the literature (26,27). In the present study, a tRNA-based genotyping study was performed for the first time in Turkey.

The amount of DNA of the organism was a key factor for the success of tRNA-based genotyping. In this study, parasite load of *E. histolytica* positive patients' isolates was determined as 10^2 and 10^3 . As a result of this, tRNA-based genotyping studies of all *E. histolytica* isolates were not performed. Determination of conventional PCR method as negative for *E. histolytica* diagnosis and highly sensitive real-time PCR results show parallelism with several studies (2–5,21) and validate our estimation about the method discussed in the sense of its being a more reliable test than the others.

The results of the molecular studies performed in the second part are as follows:

a) It is thought that real-time PCR was more feasible because of its determination of 2 amoeba species (*E. histolytica* and *E. dispar*) in a single tube and having high sensitivity (single tube real-time PCR method provides determination of parasite load in the concentration of 10^2 copies/mL) because conventional PCR has sensitivity of 10^3 copies/mL.

b) In 55 individuals with diarrhea, *E. histolytica* was detected in 20 individuals while *E. dispar* was detected in the remaining. However, in 29 asymptomatic individuals, *E. histolytica* and *E. dispar* were detected in 2 and 27 of them, respectively. As a result, while *E. histolytica* causes diarrhea as a symptom, it was worthy of note that *E. dispar* was determined at an almost equivalent rate in individuals having diarrhea as well as in asymptomatic individuals. It was expected that symptom formation such as diarrhea was high with respect to number of copy/mL in terms of parasite load intensity according to the clinical results. Diarrhea was determined more in samples diagnosed with *E. histolytica* by real-time PCR clinically; in addition, although amoeba load was determined as 10^3 copy/mL most frequently in diarrhea patients (17 of 20 patients), there was no significant difference in terms of copies number. The majority (22 of 35) of the diarrhea patients in which *E. dispar* was detected by real-time PCR had a parasite load of 10^3 and 10^4 copies/mL. Although similar (10^3 copies/mL) or higher parasite loads were detected for *E. dispar* by PCR in asymptomatic individuals, it was observed that this finding was not associated with the disease process.

c) Three different genotypes were defined in 16 isolates by using 6 loci after tRNA-based genotyping was performed. *E. histolytica* was determined in approximately 2.6% of diarrhea/asymptomatic individuals via real-time PCR, and so tRNA genotyping of these gave 3 different genotypes. Existence of isolates having different genotypes even in close geographical location can cause formation of symptoms in different ways. Due to the limited amount of pure parasite DNA recovery after extraction in direct parasite detection, it must be considered that DNA recovery after cultivation, instead of direct testing, would allow increasing the number of genotypes. This was confirmed by the existence of these kinds of problems regarding tRNA

analysis in other studies (26,27). In a study involving a tRNA-based method, 3 genotypes were determined by using 3 STR loci (D-A, A-L, and R-R) in 6 samples in Turkey and it was observed that these genotypes are different than ones in Georgia (28). In a study by Ali et al., 16 isolates from Bangladesh, 1 from Italy, and 1 from the USA were examined and they found that D-A, A-L, and S-D loci were successful, N-K2 and R-R loci were less successful, and S-Q locus was unsuccessful due to multiple bands (27). In a Japanese study, 8 genotypes were observed in clinical isolates from 12 diarrhea/dysenteric patients and a genotype was discovered having similarity with that from asymptomatic cases (26). As with other intestinal protozoa, molecular detection and genotyping are also important for *E. histolytica* (29,30).

In a study performed in a certain region of Turkey with a relatively low number of isolates, it was concluded that there might exist different genotypes and these genotypes could play a role in diarrhea formation; the parasite load of 10^2 and higher may contribute to diarrhea formation but there was no certain rule for this; and finally there might be asymptomatic individuals having higher loads of parasite as observed only for 1 individual in this study. There was another conspicuous point in the present study that conventional PCR has some limitations in cases of low parasite load in some individuals and so there might be some problems in diagnosis and treatment. Because of these reasons,

highly sensitive and specific methods such as real-time PCR should be regarded as the reference test for an accurate and reliable diagnosis. Another important point was that symptoms such as diarrhea were observed in some *E. dispar* infected individuals. This indicated the necessity for a detailed *E. dispar* genotyping based investigation contributing to amoebiasis pathogenesis (31).

In conclusion, there are different strains in the same region and this may affect clinical aspects (diarrhea). As a consequence, immensely large scale, deliberate and organized molecular epidemiological studies are required not only to determine the reason for asymptomatic or symptomatic (diarrhea or dysentery) amoebiasis but also for diagnosis and to understand the pathogenesis and virulence of *E. histolytica* strains showing high degrees of heterogenetic causes.

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