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Genetic Variability of the Serine-Rich Gene of *Entamoeba histolytica* in Clinical Isolates from Turkey*

Mehmet TANYÜKSEL[1]
Mustafa ULUKANLİGİL[2]
Hasan YILMAZ[3]
Zeynep GÜÇLÜ[1]
R. Engin ARAZ[1]
Gürkan MERT[4]
Özgür KORU[1]
William A. PETRI, Jr[5]

[1] Division of Medical Parasitology, Department of Microbiology and Clinical Microbiology, Gülhane Military Medical Academy, 06018 Etlik, Ankara - TURKEY

[2] Department of Microbiology, Faculty of Medicine, Harran University, Şanlıurfa - TURKEY

[3] Department of Parasitology, Faculty of Medicine, Yüzüncü Yıl University, Van - TURKEY

[4] Turkish Armed Forces Medical Commander, Infectious Disease Officer, Ankara - TURKEY

[5] Departments of Internal Medicine, Microbiology, and Pathology, Virginia University, Charlottesville, VA 22908-1340, USA

Aim: The spectrum of clinical intestinal disease due to *Entamoeba histolytica* infection ranges from an asymptomatic carrier state to fulminant colitis with an array of manifestations. The extent of strain diversity for *E. histolytica* and the extent to which strains differ in virulence are not clearly known. In this study, we aimed to investigate the genetic diversity of *E. histolytica* isolates from Turkey.

Materials and Methods: In order to study genetic diversity, the serine-rich gene of *E. histolytica* (SREHP) was investigated in 26 clinical isolates from Turkey by nested PCR amplification and restriction enzyme fragment length polymorphism (RFLP) analysis.

Results: Twelve distinct DNA patterns were observed after AluI digestion of nested PCR products. The results demonstrate an extensive genetic variability among Turkish *E. histolytica* clinical isolates.

Conclusions: Genotyping seems to be a useful epidemiologic tool to identify common-source outbreaks as well as strain-specific manifestation of an infection.

Key Words: *E. histolytica*, SREHP, serine rich *E. histolytica* protein, PCR, genetic diversity

Türkiye'de Klinik İzolatlarda *Entamoeba histolytica* Serinden Zengin Genin Genetik Farklılığı

Amaç: *Entamoeba histolytica* enfeksiyonlarının neden olduğu klinik intestinal hastalık yelpazesinde asemptomatik taşıyıcılıktan fulminan kolite kadar değişik tablolar görülmektedir. *E. histolytica* suş ya da şuşlar arası farklılığın virulans üzerine etkisi açıkça bilinmemektedir. Çalışmamızda Türkiye'den elde edilmiş *E. histolytica* izolatlarda genetik farklılığı araştırmayı amaçladık.

Yöntem ve Gereç: Genetik farklılığı çalışabilmek amacıyla Türkiye'den elde edilmiş 26 klinik izolatta, sisteinden zengin *E. histolytica* geni (SREHP), PZR amplifikasyon ve restriksiyon enzim parçacığı uzunluk polimorfizmi (RFLP) analizleriyle araştırılmıştır.

Bulgular: Nested PZR ürünlerinin AluI enzimi ile kesilmesi sonrasında 12 farklı DNA paterni bulunmuştur. Sonuçlar *E. histolytica* klinik izolatları arasında yoğun genetik farklılığı göstermektedir.

Sonuç: Genotiplendirme, enfeksiyonlarda ortaya çıkan suşa özgü klinik tablolarda olduğu kadar, toplum kaynaklı salgınları tanımlamada da yararlı epidemiyolojik araç olarak değerlendirilebilir.

Anahtar Sözcükler: *E. histolytica*, SREHP, sisteinden zengin *E. histolytica* protein, PZR, genetik farklılık

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Correspondence
Mehmet TANYÜKSEL
Division of Medical Parasitology, Department of Microbiology and Clinical Microbiology, Gülhane Military Medical Academy, 06018 Etlik, Ankara - TURKEY

tanyuksel@hotmail.com

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Introduction

Amebiasis is a common parasitic disease caused by *Entamoeba histolytica*. It is estimated that about 10% of the world's population is infected by the closely related parasites *E. histolytica*, *E. dispar*, and *E. moshkovskii.* Over 50 million cases of invasive disease and more than 100,000 deaths occur worldwide annually (1). Infections commonly caused by the non-pathogens *E. dispar* and *E. moshkovskii* are approximately 10-fold more frequent than infection caused by invasive *E. histolytica* (2). The indistinguishable morphology of *E. histolytica, E. dispar*, and *E. moshkovskii* makes it very important to diagnose amebiasis using *E. histolytica*-specific tests such as the TechLab antigen detection EIA test (*E. histolytica* II test) or polymerase chain reaction (PCR) (3). In recent years, several studies have been performed to describe parasite diversity in various communities and a number of molecular methods have been developed for describing and comparing complex microbial communities (4).

The genetic diversity of *E. histolytica* in an endemic population is reflected in SREHP gene polymorphism and the SREHP locus shows considerable genetic variation (5,6). Previous studies have shown 10 patterns of SREHP polymorphism among 18 isolates from different geographic areas (6). Characterization of additional isolates is needed to determine if a distinct subset of strain is associated with asymptomatic infections, amebic dysentery, and amebic liver abscess (5,6). In this paper we report the genetic diversity of *E. histolytica* isolates from stool specimens determined by nested PCR of the SREHP gene.

Materials and Methods

Stool specimens. A total of 26 (25 stool plus 1 abscess material) specimens were collected from patients: 18 with diarrhea, 3 with dysentery, 1 with an amebic liver abscess, and 4 from asymptomatic individuals (non-diarrhea/dysentery) examined in Van and Şanlıurfa, in the eastern and southeastern regions of Turkey, respectively.

Stool samples were examined for ova and parasites at the Microbiology and Parasitology Laboratories of Harran University and Yüzüncü Yıl University. Microscopically, each stool specimen was examined in fresh saline, Lugol's iodine, and permanent stain (trichrome staining) preparations.

Stool examinations by modified acid-fast staining showed no parasites such as *Cryptosporidium* spp., *Cyclospora cayetanensis*, or *Isospora belli*. Stool specimens were cultured on various selective media for members of *Enterobacteriaceae.* However, pathogenic bacteria (*Salmonella, Shigella* etc.) were not isolated.

All stool specimens were also tested for the presence of *E. histolytica* by fecal antigen detection. Antigen detection testing of stool specimens was performed with the *E. histolytica* II enzyme immunoassay (EIA) test (TechLab Inc., Blacksburg, VA, USA). Briefly, assay microtiter wells were incubated with 1 drop of monoclonal antibody (mAb)-enzyme conjugate and 200 µl of diluted stool specimen for 2 h at room temperature as described by Tanyuksel et al. (7). The contents of the well strips were then shaken and washed 5 times. Residual liquid was then removed by striking the strip once against a paper towel, 2 drops of substrate were added to all test wells, and the strip was incubated at room temperature for 10 min. After adding 1 drop of stop solution, the optical density (OD) at 450 nm was measured with an automatic microplate spectrophotometer (Bio-Tek Instruments Inc., USA). A positive result was defined as an OD reading >0.05 after subtraction of the negative control OD.

PCR for amplification of small subunit ribosomal RNA (SSU rRNA) gene of *E. histolytica* was performed on the entire stool derived DNA prior to amplification of the SREHP gene. The SREHP gene was successfully amplified from 25 stool samples and the single liver abscess sample examined.

DNA purification from stool/liver abscess. Extraction of DNA from the stool/liver specimens was carried out with 0.2 g specimens using the QIAamp DNA stool mini kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. Lastly, supernatants were transferred to fresh tubes and the nucleic acids were collected by spinning for 15 min at 4 °C and washed with 70% ethanol. Dry pellets were dissolved in 30 µl of TE buffer.

Nested small subunit rRNA PCR amplification. The isolates were characterized by amplifying and analyzing the SSU rRNA PCR. The initial and nested PCRs were performed as described by Haque et al. (8). Briefly, for SSU rRNA PCR, the initial primer set Ef (5'-ATC TGG TTG

ATC CTG CCA GT-3') and Er (5'-ATC CTT CCG CAG GTT CAC CT-3') was used. The nested primer set (Ehf/Ehr) amplifies the SSU rRNA genes of both *E. histolytica* (Ehf: 5'-GGC CAA TTC ATT CAA TGA ATT GAG-3' and Ehr: 5'-CTC AGA TCT AGA AAC AAT GCT TCT C-3'). After a freshly made "master mix" (5 ml of 10X PCR buffer, 2 ml of dNTP, 0.5 ml of Hot Star Taq, 1 ml of forward primer, 1 ml of reverse primer, and 39.5 ml of water) was prepared, PCR cycling was performed in a DNA thermal cycler (Primus MWG Biotech AG, Ebersberg, Germany) with an initial 95 °C incubation of 2 min for Ehf/Ehr, followed by 30 cycles of 1 min at 94 °C, 1 min at 47 °C, and 2 min at 72 °C, and a final extension cycle of 7 min at 72 °C. Amplified PCR products were analyzed on a 1% agarose gel in 1X Tris-boric acid-EDTA buffer (TBE) and stained with 0.2 mg/ml of ethidium bromide (Sigma).

Nested SREHP gene PCR amplification. PCR amplification of the SREHP gene was accomplished as described previously by Ayeh-Kumi et al. (5) using primers (initial SREHP-5: 5'-GCT AGT CCT GAA AAG CTT GAA GCT G-3' and SREHP-3:5'-GGA CTT GAT GCA GCA TCA AGG T-3') and (nested SREHP-5: 5'-TAT TAT TAT CGT TAT CTG AAC TAC TTC CTG-3' and nested SREHP-3: 5'-TGA AGA TAA TGA AGA TGA TGA AGA TG-3'), based on the nucleotide sequence of the SREHP gene of HM1-IMSS strain. Initial primers yielded a 549-bp fragment whereas nested primers amplified a 450-bp fragment for HM-1:IMSS strain.

The initial PCR "master mix" included 2.5 ml of 10X PCR buffer, 0.2 ml of dNTP, 0.3 ml of Hot Star Taq, 0.6 ml of forward primer, 0.6 ml of reverse primer, 2.6 ml of $MgCl_2$, and 17.2 ml of water. Cycling was performed in a DNA thermal cycler (Primus MWG Biotech AG, Ebersberg, Germany) and consisted of an initial 15 min incubation at 95 °C, followed by 30 cycles each of 1 min at 94 °C, 1.5 min at 50 °C, and 2 min at 72 °C, and a final extension cycle of 7 min at 72 °C. This step was followed by nested PCR using 1 ml of a 1:50 dilution of the initial PCR product as the template DNA. Only the annealing temperature was changed (from 50 to 55 °C) for the nested PCR. All reagents were obtained from QIAgen, Hilden, Germany.

AluI digestion of the nested SREHP PCR products. The PCR products were additionally analyzed by digestion with the restriction endonuclease *AluI* (Gibco BRL, Life Technologies). Later, 7 ml of nSREHP PCR product was digested for 2 h at 37 °C according to the manufacturer's recommended procedure. Digested PCR products were electrophoresed in 2% agarose gels, in TBE buffer (100 mM Tris–HCl, 90 mM boric acid, 1 mM Na_2EDTA), ethidium bromide solution 10 mg/ml, at 100 V for 1 h. The gel was visualized under UV light and photographed.

Ethical approval and informed consent. This study was reviewed and approved by the Human Investigation Committee of the University of Virginia and the IRB (local ethics committee). Permission to work in the community was obtained as individual written informed consent from subjects, parents, or guardians. Subjects enrolled in the study were from Van and Şanlıurfa, Turkey.

Results

The nested SREHP PCR and *AluI* RFLP analyses were used to investigate genetic diversity among 26 isolates of *E. histolytica* from different geographical origins. We detected both size and restriction site polymorphisms among the isolates from within these endemic areas, Şanlıurfa and Van. The nested SREHP PCR amplification resulted in the appearance of the characteristic 450 bp band in 16 of 26, with additional lighter –150, 300, 600, and 700 bp bands found also in most samples (Figure 1). *AluI* restriction enzyme digestion of the nSREHP products revealed different patterns with 180, 250, 300, and 450 bp bands (data not shown). Altogether, restriction enzyme digestion of the PCR products yielded 12 distinct DNA banding patterns among the 26 specimens (Table).

Discussion

Intraspecies genetic diversity has been demonstrated to be important in the pathogenesis and epidemiology of several pathogens, such as *Salmonella* (9). It is also important to consider strain-to-strain variation when identifying antibiotic sensitivities and vaccine antigens and when developing tools for molecular diagnostics. Here, we present a description of the variability in *AluI* digested nested SREHP gene expression patterns among 26 clinical isolates of *E. histolytica*.

Table. Origin of intestinal/amebic liver abscess isolates of *Entamoeba histolytica* and nSREHP PCR/digestion results from stool/abscess isolates in Turkey.

No. of sample	Isolate No.	Origin	Sample	Microscopy	PCR for *E. histolytica* (ssurRNA)	ELISA-antigen specific *E. histolytica*	nSREHP (bp)	SREHP+ AluI (bp)	Type / Pattern
1.	# HU-04-17	Diarrhea	Stool	Negative	Positive	Positive	450	250	1
2.	# HU-04-105	Diarrhea	Stool	Negative	Positive	Positive	450	250	1
3.	# HU-04-83	Diarrhea	Stool	Negative	Positive	Positive	450	250	1
4.	# HU-04-74	Diarrhea	Stool	Positive	Positive	Positive	450	250	1
5.	# HU-04-110	Non-diarrhea/dysentery	Stool	Negative	Positive	Positive	450	180	2
6.	#Van-03-131	Diarrhea	Stool	Negative	Positive	Positive	450	250	1
7.	#Van-03-114	Dysentery	Stool	Negative	Positive	Positive	450	250	1
8.	#Van-AKA-01	ALA	Abscess fluid	Positive	Positive	Positive	450	180	2
9.	#Van-03-141	Diarrhea	Stool	Positive	Positive	Positive	450	250	1
10.	#Van-03-142	Non-diarrhea/dysentery	Stool	Positive	Positive	Positive	450	250	1
11.	#Van-03-151	Diarrhea	Stool	Positive	Positive	Positive	450	250	1
12.	#Van-03-118	Diarrhea	Stool	Negative	Positive	Positive	450	250	1
13.	#Van-03-112	Diarrhea	Stool	Positive	Positive	Positive	450&600 $^{\text{double band}}$ &700	250	3
14.	#Van-03-122	Diarrhea	Stool	Negative	Positive	Positive	600 $^{\text{double band}}$	300&450	4
15.	#Van-03-111	Diarrhea	Stool	Negative	Positive	Positive	600 $^{\text{double band}}$	300&450	4
16.	#Van-03-108	Non-diarrhea/dysentery	Stool	Negative	Positive	Positive	450	250	1
17.	#Van-03-87	Diarrhea	Stool	Positive	Positive	Positive	300 $^{\text{double band}}$	250	5
18.	#Van-03-92	Diarrhea	Stool	Negative	Positive	Positive	300&600	300	6
19.	# HU-04-09	Diarrhea	Stool	Positive	Positive	Positive	170 & 450	180	7
20.	# HU-04-97	Non-diarrhea/dysentery	Stool	Negative	Positive	Positive	150	250	8
21.	# HU-04-58	Diarrhea	Stool	Positive	Positive	Positive	300 $^{\text{double band}}$ &700$^{\text{faint}}$	300 $^{\text{double band}}$	9
22.	# HU-04-62	Diarrhea	Stool	Negative	Positive	Positive	300	300 $^{\text{double}}$	10
23.	# HU-04-84	Diarrhea	Stool	Negative	Positive	Positive	150	250	8
24.	# HU-04-160	Diarrhea	Stool	Negative	Positive	Positive	150&180&450	120&180&250	11
25.	#Van-03-90	Dysentery	Stool	Negative	Positive	Positive	300 $^{\text{double band}}$ & 600	250	12
26.	#Van-03-85	Dysentery	Stool	Negative	Positive	Positive	600	300 & 450	4

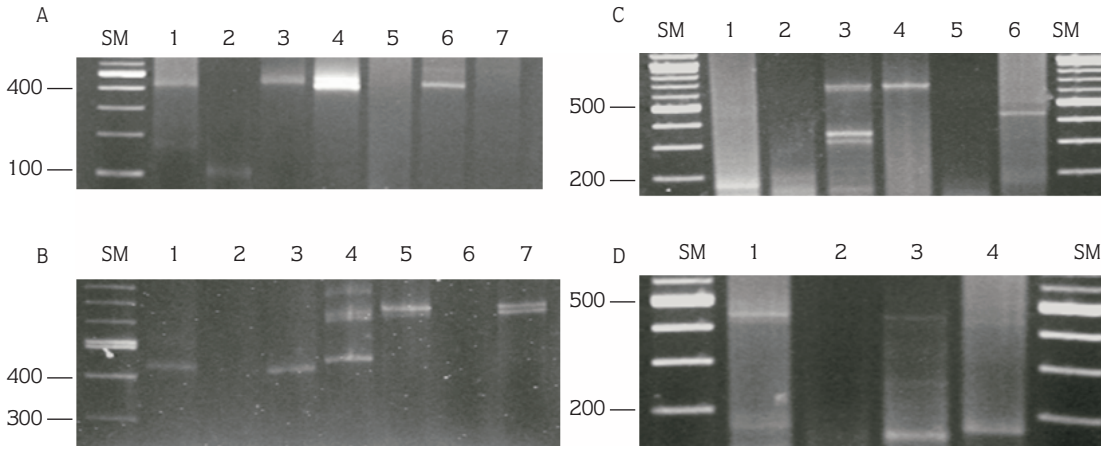

Figure 1. Gel photographs of nested SREHP PCR products amplified from DNA of *E. histolytica*-specific antigen-positive samples (from stool isolates).

Lanes 1-6 represent test isolates: (A) 1- positive control, 2- HU 04-97, 3- Van 03-108, 4-Van 03-131, 5- Van 03-127, 6- Van 03-114, 7- Negative control; (B) 1- positive control, 2- negative control, 3- Van-03-118, 4- Van-03-112, 5- Van-03-122, 6- Van-03-124, 7- Van-03-111; (C) 1- HU 04-97, 2-Van 03-159, 3- Van-03-90, 4- Van 03-85, 5- negative control, 6- positive control; (D) 1- HU 04-160, 2-HU 04-159, 3-HU 04-09, 4- HU 04-84.

SM is the 100-bp DNA ladder (Gibco BRL). PCR products were separated electrophoretically in a 2% agarose gel.

Our work demonstrated both genetic diversity within a geographic region, and genetic differences between geographic regions of Turkey. We detected mostly SREHP polymorphism pattern #1 (7 of 15) in the Van geographic area, with the other patterns #2, 3, 4, 5, 6, and 12. According to symptoms, 4 of 9 diarrheal individuals were pattern #1. Although a dysenteric patient and 2 asymptomatic individuals were pattern #1, the fact that most of the patients with diarrhea were also pattern #1 indicated a possible correlation between SRHEP polymorphism and clinical outcome. The sole patient with amebic liver abscess had pattern #2. In Şanlıurfa, 4 of 11 clinical isolates, all with diarrhea, were pattern #1. Other patterns from this region were different: #7, 8, 9, 10, and 11. One of 2 asymptomatic individuals was pattern #2; the other was pattern #8. It is not possible to assign a genotype in every specimen because of a technical problem; in some specimens, although the SREHP gene was successfully amplified, the *Alu*I restriction enzyme digestion of the PCR products revealed faint or no bands.

Extensive genetic diversity of *E. histolytica* by analysis of the SREHP gene by PCR amplification and *Alu*I digestion was also observed recently in Bangladesh and Georgia (5,10). Ayeh-Kumi et al. (5) found the existence of a high level of SREHP genetic diversity among isolates of *E. histolytica* (34 distinct DNA patterns among 54 isolates of *E. histolytica* evaluated) within a "restricted" endemic area, demonstrating that polymorphism is extensive within a single geographic region. Haighighi et al. were also able to demonstrate that this parasite from an area of endemicity in Southeast Asia had an extremely polymorphic genetic structure, e.g., 21 different combinations of genotypes were found among the 27 isolates obtained from Thailand (11). The constancy of the SREHP polymorphisms over time (12) allows for outbreak investigations as well as correlation of virulence to genotype. Our study recommends the need for further intensive research and evaluation of molecular typing results with epidemiological background. We conclude that the present study, as well as the work of others cited above, demonstrates that SREHP gene markers can be considered robust molecular markers for population genetics, epidemiological, and ecological studies of the invasive pathogen *E. histolytica*.

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