

Tehran University of Medical Sciences Publication http:// tums.ac.ir

Iranian J Parasitol

Open access Journal at http://ijpa.tums.ac.ir



Iranian Society of Parasitology http://isp.tums.ac.ir

Original Article

Sequence Diversity in tRNA Gene Locus A-L among Iranian Isolates of Entamoeba dispar

^{*}E Nazemalhosseini-Mojarad¹, M Azimirad¹, Z Nochi¹, S Romani¹, M Tajbakhsh¹, M Rostami-Nejad¹, A Haghighi², MR Zali¹

 ¹ Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
² Department of Medical Parasitology & Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

(Received 22 Jul 2011; accepted 25 Nov 2011)

ABSTRACT

Background: A number of methods for detecting diversity in *Entamoeba* have been described over the years. In the present study the genetic polymorphism of noncoding locus A-L was analyzed using PCR and sequencing in order to clarify the genotypic differences among *E. dispar* isolates.

Methods: A total of 28 *E. dispar* from patients with gastrointestinal symptoms were determined and the genomic DNA was extracted directly from stool. For genotype analysis; Locus A-L was amplified by PCR and PCR products were sequenced .The sequences obtained were edited manually and aligned using Gene Runner software.

Results: With sequencing of PCR products a reliable genetic diversity in size, number and position of the repeat units were observed among the Iranian *E. dispar* isolates in locus A-L gene. Sequences showed variation in length from 448bp to 507bp and seven distinct types were identified. **Conclusion:** The genetic diversity of loci like A-L shows them to be suitable for epidemiological studies such as the characterization of the routes of transmission of these parasites in Iran.

Keywords: Entamoeba dispar, STRs, Locus A-L, Diversity, Iran

*Corresponding author: Fax: + 98 21 22432517, E-mail:ehsanmojarad@gmail.com

Introduction

number of methods for detecting the diversity in Entamoeba have been described over the years (1), but recently Ali et al. investigated the association between the genotypes of parasites and the clinical outcome of infection (2) using a 6-locus genotyping system based on tRNA-linked short tandem repeats (STRs) (3). The six targets for amplification in this method (Loci S-Q, D-A, A-L, S^{TGA}-D, R-R and N-K2) were selected from among over 40 STR-containing loci linked to tRNA genes in E. histolytica (1). One of these polymorphic loci is Locus D-A, previously called locus 1-2, has been shown to be potentially useful for investigating the molecular epidemiology of E. histolytica and *E. dispar* (4, 5).

Zaki et al. isolated and characterized locus D-A, and later compared the nucleotide sequence of this locus between *E. dispar* and *E. histolytica*. This revealed significant differences in both the STRs and the flanking regions (6,7). Haghighi et al. analyzed the genetic polymorphisms of four loci, including D-A, 5-6 among 79 isolates of *E. histolytica* obtained from different geographic regions. They reported large scale genetic differentiation between Japanese and Thai isolates (4,8). Recently, molecular studies have been extended to distinguish and investigate the distribution of these two species in Iran (9-11).

In the present study, genetic polymorphism of another tRNA-liked STR-containing locus, A-L, was analyzed using PCR and sequencing methods in order to clarify further the genotypic differences among *E. dispar* isolates.

Materials and Methods

A total of 28 *E. dispar* strains were analyzed. Twenty four of them were collected from Iranian patients referred to the clinical laboratories of hospitals in the city of Tehran and Zahedan and 4 strains were collected from asymptomatic individuals referred to health care centers in the city of Gonbad (5). Clinical information on the samples is given in Table 1. All the samples used in this study were diagnosed as positive for *Entamoeba* spp. by microscopic examination of fresh stools using direct smears, formalinether concentrated, and trichrome stained specimens.

The genomic DNA was extracted directly from stool and samples were identified to species level by locus D-A based PCR analysis, as previously described (5). For genotype analysis, Locus A-L was amplified by PCR with the primer set 5'-CATCTCCAT TATTATGTATCTATTTATC-TATTTA-3'and 5'- GGCACGAATGCTTTGA-TATATAA-3' (3). PCR products were analyzed by electrophoresis using 1.8% agarose gels (Fermentas, #R0491) in Tris-boric acid-EDTA buffer containing ethidium bromide after which the gels were photographed under ultraviolet light (UVIdoc, UVItec Limited, Cambridge, United Kingdom). The PCR products were sequenced using the amplification primers and an Applied Biosystems (ABI) Terminator Cycle Sequencing Ready Reaction kit (BigDye® Terminator V3.1 Cycle Sequencing Kit) on an ABI 3130xl Genetic Analyzer. The sequences obtained were edited manually and aligned using Gene Runner software (version 3.05). Nucleotide sequences, except forward and reverse primer regions, were aligned with the only previously available locus A-L sequence from E. dispar in GenBank (AY842969). All sequences were submitted to the GenBank/EMBL/DDBJ database under accession numbers HO439931-HQ439958.

Results

In order to investigate genetic diversity, the PCR-amplified products from 28 *E. dispar* isolates were subjected to direct sequencing. Samples were sequenced in both directions and, when any variations were found, results were confirmed by sequencing of at least two independent PCR products. All sequences were analyzed by Chromas version 1.45 (Technelysium, Queensland, Australia) and the sequence homology was compared with the sequences in GenBank by BLAST analysis. PCR amplification and sequencing of the PCR products showed a remarkable level of genetic diversity in size, number and position of the repeat units among the *E. dis*-

par isolates (Fig. 1). Nucleotide sequence length varied from 448bp to 507bp which led to visible differences in PCR product size (Table 1). Seven distinct nucleotide sequences were obtained from the isolates while gel analysis of the PCR products show three groups distinguishable by size. Sequence E represents the dominant genotype (11/28, 37%) among the Iranian isolates and its 472 bp fragment was also the most frequent size found. The STR organization in locus A–L from *E. dispar* SAW760 (AY842969), which has 507 nucleotides, is compared to the organization in sequences from the Iranian isolates in Fig. 1.

No.	Isolates	olates Isolation		Clinical	Sex	Age	Size of	Accession	Туре	Туре
		date	loca-	symptoms ^a		(yr)	PCR frag-	numbers	Locus	Locus
			tion				ments (bp)		A-L	D-A (5)
1	NH1I	2006	Tehran	Abdominal	F	20	449	HQ4399	F	IV
	R			pain,				31		
				diarrhea						
2	NH2I	2006	Tehran	Abdominal	Μ	6	449	HQ4399	F	VII
	R			pain				32		
3	NH3I	2006	Tehran	Abdominal	Μ	22	449	HQ4399	F	II
	R			pain,				33		
				bloating						
4	NH4I	2006	Tehran	Abdominal	Μ	32	499	HQ4399	В	Ι
	R			pain				34		
5	NH5I	2006	Tehran	Abdominal	F	27	507	HQ4399	А	Ι
	R			pain,				35		
				vomiting						
6	NH6I	2006	Tehran	Abdominal	Μ	63	472	HQ4399	Е	VI
	R			pain				36		
7	NH7I	2007	Tehran	Diarrhea,	Μ	33	483	HQ4399	С	IV
	R			bloating				37		
8	NH8I	2007	Tehran	Abdominal	F	24	483	HQ4399	С	III
	R			pain,				38		
				diarrhea						
9	NH9I	2007	Tehran	diarrhea	F	36	499	HQ4399	В	VI
	R							39		
10	NH10I	2007	Tehran	Abdominal	F	38	507	HQ4399	А	III
	R			pain				40		

Table 1: Background and genotype of E. dispar isolates

11	NH11I	2007	Tehran	Abdominal	F	63	499	HQ4399	В	III
	R			pain,				41		
		••••	-	bloating			100		~	
12	NH12I	2007	Tehran	Abdominal	Μ	64	483	HQ4399	C	Х
12	K NII12I	2007	Tahman	pain	М	40	507	42	٨	V
13	NH131	2007	Tenran	Abdominal	M	42	507	HQ4399	А	V
14	К NH1/I	2007	Tahran	Abdominal	м	54	183	43 HO4300	р	т
14	R R	2007	Teman	nain	IVI	54	405	11Q4399 44	D	1
	K			vomiting						
15	NH15I	2007	Tehran	Abdominal	М	53	472	HO4399	Е	Х
10	R	2007		pain		00	.,_	45	-	
16	NH16I	2007	Tehran	Abdominal	F	8	448	HO4399	G	IV
	R			pain,				46		
				bloating						
17	NH17I	2007	Tehran	Diarrhea, vo-	Μ	14	472	HQ4399	Е	Ι
	R			miting				47		
18	NH18I	2007	Tehran	Abdominal	F	12	472	HQ4399	Е	III
	R			pain				48		
19	NH19I	2007	Tehran	Abdominal	F	20	499	HQ4399	В	Х
	R			pain,				49		
20	NU1201	2007	T 1	vomiting	Б	21	100	110 1200	ъ	V
20	NH201	2007	Tehran	Abdominal	F	31	499	HQ4399	В	Х
	ĸ			pain,				50		
21	NH211	2007	Tehran	Abdominal	F	8	507	HU1300	Δ	v
21	R R	2007	reman	nain	1	0	507	51	п	Λ
	K			diarrhea				51		
22	SHN3I	2004	Zahe-	Abdominal	F	25	472	HO4399	Е	IX
	R	2001	dan	pain	-		.,_	56	-	
23	SHN4I	2004	Zahe-	Abdominal	Μ	42	472	HQ4399	Е	IX
	R		dan	pain,				57		
				vomiting						
24	SHN7I	2004	Zahe-	Abdominal	Μ	32	472	HQ4399	Е	XII
	R		dan	pain,				58		
				vomiting						
25	NHM1	2005	Gon-	Asymptomat	F	28	472	HQ4399	E	XI
	IR	2005	bad	ic			470	52	F	
26	NHM2	2005	Gon-	Asymptomat	Μ	31	472	HQ4399	E	VII
27		2005	bad	1C	м	21	470	53	Б	VII
21	INHM3	2005	G0n-	Asymptomat	IVI	31	4/2	HQ4399	Ľ	VII
28	IK NHM4	2005	Gon	IC Asymptomet	м	31	172	J4 HO4300	Б	VI
20	INTINI4 IR	2005	bad	ic	141	51	+/2	55	Ľ	ЛІ
	ш\		Juu	10				55		

Table 1: Continued ...



Fig. 1: Schematic representation of the STR polymorphisms in locus A-L of *E. dispar*. The 7 distinct sequence types are shown as well as the identification tag for the isolates that matched each type; also shown is the structure of locus A-L sequence in the standard isolate, *E. dispar* SAW760 (AY842969). The sequences of each of the nine repeat types are shown beside their corresponding colored block. The conserved non-repeated regions are shown as a single line

Nazemalhosseini et al.: Sequence Diversity in tRNA Gene Locus A-L ...

Discussion

The ability to identify strains of Entamoeba dispar may lead to insights into the population structure and epidemiology of the organism. When polymorphism in two E. histolytica loci, 1-2 and 5-6, was studied in 2001 by Zaki et al., the remarkable diversity in length, type and numbers of the repeat units found showed that they have the potential to allow the investigation of genetic differences between invasive and noninvasive E. histolytica isolates. Sequences corresponding to the polymorphic loci reported from E. histolytica have also been detected in E. dispar. Comparison of nucleotide sequences in two loci between E. dispar and E. histolytica revealed significant differences in both the repeats and the flanking regions, which allowed the typing and differentiation of these two parasites simultaneously (6,7). However although variation in locus A-L has been investigated to some extent in E. histolytica (12), it has not been investigated previously in E. dispar. The tRNA gene regions in locus A-L are conserved and are the site of the primers used, but in the middle there are repeat units of between 8 and 15 nucleotides which vary among isolates. Elimination, duplication and substitution of units in this repeat-containing region are the basis of polymorphisms detected in the two species.

In our previous study PCR amplification of locus D-A among Iranian *E. dispar* isolates, showed a remarkable genetic diversity in size and this result confirmed by Sequencing of PCR products (5). By simultaneous investigation of locus A-L and locus D-A (5), 26 subtypes out of 28 *Entamoeba dispar* isolates were distinguished (the molecular patterns of NH19IR and NH20IR, also NHM2IR and NHM3IR are not different in two loci) (Table 1). In this study, no meaningful correlation between infection with E. dispar and age, sex or parasite genotype was observed. However, it appears that sequence type E is overrepresented in the male individuals compared to females (out of 11 individuals who showed this type, 8 of these were males) or sequence type E is common in asymptomatic patients. In 2001, in Bangladesh, the role of genetic diversity in E. histolytica virulence was studied and it was clarified that the genetic diversity of E. histolytica subspecies in endemic regions is because of SREHP polymorphism. Noticeably, the polymorphism of liver amebiasis subspecies was different from intestinal amebiasis subspecies (13-14). Haghighi et al. reported a considerable polymorphic in size, number and position of the repeat units in four loci (1-2, 5-6, SREHP and Chitinase) of different E. histolytica isolates obtained from stool samples of mentally handicapped individuals and male homosexuals from different regions of Japan (4, 8). They proposed that genotyping of ameba isolates should help to determine geographic origins of isolates and routes of transmission. Although the studies of Haghighi et al. did not detect a link between genotype and symptoms, their samples were from geographically diverse sources and acquired over a number of years (4, 8).

The studies of Ali et al. developed a reliable method for PCR-based genotyping of *E*. *histolytica* based on variation in the numbers of short tandem repeats that are linked to tRNA genes in this species and suggesting that the parasite genome does contribute in some way to the outcome of infection with *E*. *histolytica* (2, 3).

In conclusion, we propose that molecular typing and analysis of genotypes of *E*. *histolytica* and *E*. *dispar* isolates from a variety of locations should help in determining

the geographic origins of isolates and routes of transmission.

Acknowledgments

The authors would like to express special thanks to Dr. C. Graham Clark from the Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, England for edition and helpful comments on this manuscript. This work was financially supported by Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, with grant number 505. The authors declare that there is no conflict of interests.

References

- Clark CG .Methods for Investigation of Diversity in *Entamoeba histolytica*. Arch Med Res. 2006; 37:258-61.
- 2. Ali IK, Mondal U, Roy S, Haque R, Petri WA- JR, Clark CG. Evidence for a Link between Parasite Genotype and Outcome of Infection with *Entamoeba histolytica*. J Clin Microbiol . 2007; 45: 285–289.
- Ali IK, Zaki M, Clark CG. Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol. 2005; 43: 5842-5847.
- 4. Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J Clin Microbiol .2002; 40: 4081-90.
- Nazemalhosseini- Mojarad E, Haghighi A, Kazemi B, Rostami -Nejd M, Abadi A, Zali MR. High genetic diversity among Iranian *Entamoeba dispar* isolates based on the noncoding short tandem repeat locus D-A. Acta Trop .2009; 111: 133-6.
- 6. Zaki M, Clark CG. Isolation and characterization of polymorphic DNA from

Entamoeba histolytica. J Clin Microbiol 2001; 39: 897-905.

- Zaki M, Meelu P, Sun W, Clark CG. Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J Clin Microbiol .2002; 40: 1271-1276.
- Haghighi A, Kobayashi S, Takeuchi T, Thammalerd N, Nozaki T. Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. J Clin Microbiol. 2003; 41: 3748-56.
- Hooshyar H, Rezaian M, Kazemi B, Jeddi-Tehrani M, Solaymani-Mohammadi S. The distribution of *Entamoeba histolytica* and *Entamoeba dispar* in northern, central, and southern Iran. Parasitol Res. 2004; 94: 96-100.
- 10. Solaymani-Mohammadi S, Rezaian M, Babaei Z et al. Comparison of a stool antigen detection kit and PCR for diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* infections in asymptomatic cyst passers in Iran. J Clin Microbiol .2006; 44: 2258-61.
- Nazemalhosseini- Mojarad E, Nochi Z, Sahebekhtiari N et al. Discrimination of *Entamoeba mos-hkovskii* in patients with gastrointestinal disorders by single-round PCR. Jpn J Infect Dis .2010; 63: 136-138.
- Escueta-Decadiza A, Kobayashi S, Takeuchi T, Tachibana H, Nozaki T. Identification of an avirulent *Entamoeba histolytica* strain with unique tRNA-linked short tandem repeat markers. Parasitol Int. 2010; 59: 75-81.
- Ayeh-Kumi PF, Ali IK, Lockhart LA, Gilchrist CA, Petri WA- JR , Haque R. *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp Parasitol.2001; 99: 80-8.
- 14. Simonishvili S, Tsanava S, Sanadze K, Chilikadze R, Miskalishvili A, Lomkatsi N, Imnadze P, Petri WA- JR, Trapaidez N. *Entamoeba histolytica*: the serine-rich gene polymorphism-based genetic variability of clinical isolates from Georgia. Exp Parasitol. 2005; 110: 313-7.