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Association between allelic variation due to short tandem repeats in tRNA gene of *Entamoeba histolytica* and clinical phenotypes of amoebiasis

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ABSTRACT

Genotypes of *Entamoeba histolytica* (*E. histolytica*) may contribute clinical phenotypes of amoebiasis such as amoebic liver abscess (ALA), dysentery and asymptomatic cyst passers state. Hence, we evaluated allelic variation due to short tandem repeats (STRs) in tRNA gene of *E. histolytica* and clinical phenotypes of amoebiasis. Asymptomatic cyst passers ($n=24$), patients with dysentery ($n=56$) and ALA ($n=107$) were included. Extracted DNA from stool (dysentery, asymptomatic cyst passers) and liver aspirate was amplified using 6 *E. histolytica* specific tRNA-linked STRs (D-A, A-L, N-K2, R-R, S-Q, and S^{TGA}-D) primers. PCR products were subjected to sequencing. Association between allelic variation and clinical phenotypes was analyzed. A total of 9 allelic variations were found in D-A, 8 in A-L, 4 in N-K2, 5 in R-R, 10 in S^{TAG}-D and 7 in S-Q loci. A significant association was found between allelic variants and clinical phenotypes of amoebiasis. This study reveals that allelic variation due to short tandem repeats (STRs) in tRNA gene of *E. histolytica* is associated different clinical outcome of amoebiasis.

1. Introduction

Amoebiasis, caused by the protozoan parasite *Entamoeba histolytica* (*E. histolytica*) include asymptomatic cyst passers, dysentery and extra-intestinal diseases (liver, lung, and rarely brain abscess). Eighty percent infected patients with amoebiaisis remain asymptomatic, remaining 20% patients develop symptomatic diseases (intestinal and extra-intestinal) (Haque et al., 2009). Mortality due to amoebiasis results mainly from extra-intestinal infections, amoebic liver abscess (ALA) being the commonest (Haque et al., 2003). Annually 50 million cases of amoebic dysentery and ALA have been reported world-wide, of whom 40,000 to 1,000,00 die (Petri et al., 2000; WHO, 1997).

Determinants of occurrence of different clinical phenotypes of *E. histolytica* infection such as asymptomatic cyst passers, dysentery and ALA still remain unclear. Genotype of the *E. histolytica* is a major determinant (Ali et al., 2007; Clark et al., 2006; Escueta-de Cadiz et al., 2010). Other factors like the host immune response may play important role (Duggal et al., 2004). Toll-like receptors (TLRs) gene polymorphism could affect the host's ability to respond to pathogens. In our previous study, we did not find any association between TLR-2 (Arg753Gln and Arg677Trp), TLR-4 (Asp299Gly and Thr399Ile) polymorphism and clinical phenotypes of amoebiasis (unpublished data). A few PCR-based DNA genotyping methods have been reported for *E. histolytica* such as serine-rich *E. histolytica* protein (SREHP) and chitinase gene (Ali et al., 2008; Ayeh-Kumi et al., 2001; Ghosh et al., 2000; Haghighi et al., 2002, 2003; Zaki et al., 2002). However these methods did not find any association between genotypes of *E. histolytica* and different clinical phenotypes.

Genome sequence of *E. histolytica* HM-1: IMSS strain revealed the presence of short tandem repeats (STRs) in tRNA gene. Repeats of A+T rich (8–16 bp) nucleotide have been most commonly reported in STRs, which leads to polymorphism (Zaki and Clark, 2001). All tRNA-linked STRs in *E. histolytica* were screened using 46 pairs of primer (Ali et al., 2005). Only 6 loci (D-A, A-L, N-K2, R-R, S-Q, and S^{TGA}-D) in tRNA gene were found to be suitable for genotyping of *E. histolytica* (Ali et al., 2007). *E. histolytica* was classified into a large number of genotypes by combination of variations in 6 loci (Ali et al., 2007; Escueta-de Cadiz et al., 2010). As the number of classes was too many, such classification does not appear to be of much clinical utility. Recently, variation in R-R locus was found to be associated with different clinical outcome of *E. histolytica* infection (Ali et al., 2012). However, studies on

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association between allelic variants in *E. histolytica* at other loci and clinical phenotypes of amoebiasis are lacking. Therefore, we aimed to evaluate allelic variation due to short tandem repeats (STRs) in tRNA gene of *E. histolytica* and clinical phenotypes of amoebiasis.

2. Methods

A total of 187 patients with amoebiasis (asymptomatic cyst passers 24, dysentery 56 and ALA 107) were included in the study over a 6-year-period (January 2006 to December 2011). Patients with ALA and dysentery were enrolled from the Department of Gastroenterology. Asymptomatic cyst passers were included from the kitchen workers and residents of the Institute. Details of patients with ALA have been reported previously (Jaiswal et al., 2012).

Liver aspirate from patients with ALA and stool samples from those with dysentery and asymptomatic cyst passers were taken. Diagnosis of ALA was based on imaging, presence of anti- amoebic IgG antibody in serum and established by the presence of amoebic DNA in liver aspirate as described previously (Jaiswal et al., 2012). Stool samples positive for the cyst and or trophozoites of *E. histolytica* on microscopy, were confirmed by the presence of *E. histolytica* lectin antigen using Tech Lab *E. histolytica* II ELISA kit (Blackburg, Virginia, USA). Stool samples were also cultured on MacConkey, deoxycholate and dextrose sorbitol rhamnose agar to look for Shigella, Salmonella and diarrhoegenic *E. coli* (Collee et al., 1996) and if positive were excluded from the study. Informed consent was obtained from all the subjects and the protocol was approved by the Institution's ethics committee (PGI/DIR/RC/957/2007).

2.1. Isolation of DNA and polymerase chain reaction (PCR)

Genomic DNA was extracted from stool samples using a QIAmp DNA stool mini kit (Qiagen Germany) as per manufacturer's instruction. DNA from liver aspirate was isolated by Cetyltrimethylammonium bromide method (Ali et al., 2005). We used species specific PCR amplification method in order to determine allelic variation in 6 loci (D-A, A-L, N-K2, R-R, S-Q, and S^{TGA}-D) as reported previously (Ali et al., 2005). The amplified PCR products were run on 2% agarose gel for further analysis (Himedia, India).

2.2. Sequencing

To know nucleotide sequences, purified PCR product were sequenced using *E. histolytica*-specific tRNA-linked STRs forward primers by fluorescent label ABI's Ampli Taq FS dye terminators on ABI model 3100. Electropherograms were analyzed and aligned with previously reported sequences of *E. histolytica* using standard software (Chromas program, Technelysium Pty Ltd, Sydney, Australia and BioEdit v7.0.5, Ibis Biosciences, Carlsbad, California, respectively). Newly identified sequences were submitted to NCBI. Association between allelic variation and clinical phenotypes (asymptomatic cyst passers, dysentery and ALA) was analyzed.

2.3. Statistical analysis

Chi-Squares test was applied to evaluate association between allelic variation and clinical phenotypes. Bonferroni correction was applied to correct for multiple comparison. Two-tailed P values <0.05 were considered significant.

3. Results and discussion

Demographic and clinical details of the patients have been included in Supplementary Table S1. Patients with ALA and dysentery were comparable in age with asymptomatic cyst passers (42 ± 16, 34 ± 16 and 36 ± 8 years respectively, P = ns). Gender distribution in the three groups was comparable.

3.1. PCR amplification and gel electrophoresis

Six polymorphic STRs loci were successfully amplified by PCR in liver aspirate samples from patient with ALA and stool from those with dysentery and asymptomatic cyst passers. A total of 9 allelic variations were found in D-A, 8 in A-L, 4 in N-K2, 5 in R-R, 10 in S^{TAG}-D and 7 in S-Q loci (fig. 1). A single band was found in the most of the samples except for 15 liver aspirate, in which a second faint band was observed near desired PCR product as shown in Fig. 1C (lane 3) and 1F (lanes 7 and 8). Since, variation in numbers and sequences of tandem repeats encoding 8- and 12-amino acids (24–36 nucleotides) in different loci of tRNA gene of *E. histolytica* have been reported (Tawari et al., 2008), both bands were subjected to sequencing.

3.2. Sequencing of amplified PCR products

Variations in nucleotide sequence were same as those observed in PCR products on agrose gel (Fig. 2). Two new sequences were identified in locus S-Q and N-K2 each. These were assigned as S1, S2 and N1, N2, respectively, and were submitted to NCBI with accession numbers JN314997–JN315000 (2011).

3.3. Frequency and types of STRs

Fig. 3 and Table 1 show allelic variation and their frequency among asymptomatic cyst passers and patients with dysentery and ALA.

3.3.1. Allelic variation in D-A locus

(a) 14 DA was mostly found among patients with ALA [asymptomatic cyst passers 2/24 (8%) vs dysentery 2/56 (3%) vs ALA 40/107 (37%), $P < 0.001$]. (b) 10DA, 11DA, 13DA, J1DA were exclusively found among asymptomatic cyst passers 22/24 (92%). (c) 5DA and 8DA were more often associated with dysentery [ALA 23/107 (21%) vs dysentery 38/56 (68%), $P < 0.001$). (d) 6DA and15DA were shared between patients with dysentery 16/56 (28%) and ALA 44/107 (41%).

3.3.2. Allelic variation A-L locus

(a) J3AL was found only in patients with ALA 11/107 (10%). (b) 4AL and J8AL were mostly found in ALA [asymptomatic cyst passer 4/24 (16%) vs dysentery 14/56 (25%) vs ALA 77/107 (72%), $P < 0.001$). (c) J1AL and J4AL were found only in dysentery 30/56 (53%). (d) J2AL was shared between dysentery 12/56 (21%) and ALA 9/107 (8%). (e) J6AL and J7AL were found only in asymptomatic cyst passer 20/24 (83%).

3.3.3. Allelic variation N-K2 locus

(a) N2 was found exclusively in patients with ALA 18/107 (17%). (b) N1 was mostly found in ALA [asymptomatic cyst passer 2/24 (8%) vs dysentery 8/56 (14%) vs ALA 89/107 (83%), $P < 0.001$). (c) 3N-K and J3N-K were shared between asymptomatic cyst passers 22/24 (92%) and patients with dysentery 48/56 (86%).

3.3.4. Allelic variation R-R locus

(a) J1RR was found only in patients with ALA 10/107 (10%). (b) 5R-R and 10 R-R were shared between patients with ALA 90/107