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GENOTYPING OF *ENTAMOEBA HISTOLYTICA* BY REAL-TIME POLYMERASE CHAIN REACTION WITH SYBR GREEN I AND MELTING CURVE ANALYSIS

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ABSTRACT

In this study, for the detection of distinct genotype of *E. histolytica* of human, a nested Real-Time PCR amplification of SREHP gene using SYBR Green I and melting curve analysis was done. A total of 60 specimens (stool and liver aspirate specimens), which were found *Entamoeba histolytica* positive by *E. histolytica* specific ELISA and ssrRNA gene PCR, were selected and the experiment was conducted during the period of July 2003 to June 2004. After melting curve analysis of amplified PCR products from these isolates of stool and liver aspirate specimens, 5 genotypes were found belonging to the melting temperatures 84°C, 83°C, 82°C, 81°C and 79°C. All these 5 genotypes were present in intestinal amoebiasis patients and when the genotypes from intestinal amoebiasis patients were compared with the genotypes of amoebic liver amoebiasis patients, the genotype 84°C melting temperature was found to be absent in amoebic liver amoebiasis patients. For both the cases of intestinal and amoebic liver amoebiasis patients the genotype belonging to 83°C melting temperature was more prevalent than the other genotypes which suggest that this genotype is more responsible for the development of amoebiasis. In comparison to conventional PCR method where we found 23 different banding patterns, the Real-Time PCR and melting curve analysis method was found to be more reliable for the detection of distinct genotypes of *E. histolytica* because with this method we found only 5 genotypes. In conclusion, this Real-Time PCR using SYBR Green I and melting curve analysis for the genotyping of *E. histolytica*, excludes the need of post PCR manipulations and would be helpful for the rapid detection and screening of *E. histolytica* genotypes among endemic population and also for the epidemiological study.

Key words: *Entamoeba histolytica*, genotype, diarrhoea, Real-Time PCR, melting curve analysis

INTRODUCTION

Entamoeba histolytica is a protozoan parasite that causes amoebic colitis and liver abscess in the people of developing countries like India and Bangladesh (Ravdin, 1995; Haque *et al*., 1997). Global statistics on the prevalence of *E. histolytica* infection indicate that 90% of infected individuals remain asymptomatic carriers while the other 10% develop clinically overt disease (Jackson *et al*., 1985; Haque *et al*., 1999). This observation might partly be due to the differences in the pathogenic potential of infecting strains (Burch *et al*., 1991) or due to the variability in the host immune response against amoebic invasion. As the variation in human immune response against amoebic infection is not clearly understood, the genetic differences among *E. histolytica* need to be determined.

Evidences of intraspecies variation among *E. histolytica* isolates were first based on isoenzyme analysis (Sargeaunt *et al*., 1978; Sargeaunt, 1988). After the failure of the isoenzyme analysis due to its less reliability and limited diversity, a polymerase chain reaction (PCR) based-strain typing methods were developed on the basis of polymorphism of different loci of *E. histolytica* including protein-coding sequences, such as those for the serine rich *E. histolytica* protein (SREHP) (Li *et al*., 1992; Kohler and Tannich, 1993) and chitinase (Ghosh *et al*., 2000), as well as non-protein-coding regions such as rRNA genes (Bhattacharya *et al*., 1992; Sehgal *et al*., 1993), a strain specific transcript (Burch *et al*., 1991), and loci 1-2 and 5-6 (Zaki and Clark, 2001).

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These polymorphic loci have been shown to be potentially useful for the determination of intraspecies variation among *E. histolytica* and also in investigating the molecular epidemiology of amoebiasis. At present, the most polymorphic gene of *E. histolytica* is that encoding the serine-rich *E. histolytica* protein (SREHP or K_2) a surface antigen with tandem 8-and 12-amino-acid repeats (Stanley *et al*., 1990; Kohler and Tannich, 1993; Ayeh-Kumi *et al*., 2001). Ayeh-Kumi *et al*. (2001) studied the genetic polymorphism among *E. histolytica* in Bangladesh using the gene encoding for SREHP and they found 34 different banding patterns out of 54 test isolates which indicates extensive intraspecies variation in *E. histolytica*.

For the study of intraspecies variation or genotyping of *E. histolytica*, different authors used PCR based typing techniques which usually require intensive and laborious post-PCR manipulation, like agarose gel electrophoresis, RFLP analysis, use of hazardous chemical, and carry a potential risk for laboratory contamination. Moreover with these methods, different authors found extensive intraspecies variations among *E. histolytica* which could not be possible to consider as distinct genotype and also difficult to observe the genotypic relation of *E. histolytica* among different types of amoebic patients. So there is a need to establish an alternative rapid method for genotyping of *E. histolytica* without the manipulation of hazardous post PCR techniques. To serve the purpose, the present study was designed for the detection of genotypes of *E. histolytica* on the basis of melting curve analysis using Real-Time PCR with SYBR Green I. The genotypes of *E. histolytica* were compared between intestinal and amoebic liver amoebiasis patients and a comparison was also studied between the results of conventional PCR method and Real-Time PCR method for genotyping of *E. histolytica.*

MATERIALS AND METHODS

This study was carried out in the Laboratory of Parasitology, Laboratory Sciences Division, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B), Mohakhali, Dhaka, Bangladesh from July 2003 to June 2004. In this study a total of 60 specimens, which were found positive to *Entamoeba histolytica* and successfully amplified the Serine-Rich *Entamoeba histolytica* Protein (SREHP) gene were selected. All these specimens have been selected depending upon different criteria, of which 50 specimens were from stools of intestinal amoebiasis patients and 10 specimens were from liver aspirate of amoebic liver amoebiasis patients from Banghabandhu Sheikh Mujibur Rahman Medical University, Dhaka. Among 50 stool specimens, 32 were from nondiarrhoeic individuals and 18 were from diarrhoeic individuals. 47 stool specimens were collected from the children of Mirpur Slum Area, Dhaka and 3 stool specimens from Matlab (Chandpur) adult subjects.

All the specimens used in this study were first examined by microscopy and with the *E. histolytica* II stool antigen detection ELISA kit (Techlab Inc., Blacksburg, VA) and then the amplification of small subunit ribosomal RNA gene of *E. histolytica* was performed for specific diagnosis. The polymorphic SREHP gene of these *E. histolytica* positive specimens was successfully amplified by using Real-Time PCR with SYBR Green I and then melting curve analysis was done for the genotyping of *E. histolytica*. Two stool specimens that were negative by microscopy, antigen detection kit, and ssrRNA gene PCR were used as negative controls. DNA isolated from an axenic culture of *E. histolytica* strain HMI:IMSS was used as the positive control for the SREHP PCR.

Extraction of DNA from stool/liver pus specimens

Trophozoites and cysts present in the stool and liver aspirates were the source of target DNA. The DNA of these specimens was extracted by using QIAamp® DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The materials and methods developed by Clark and Diamond (1993) were used for the extraction of DNA from the culture specimen of *E. histolytica*.

Nested small subunit rRNA gene PCR

Two sets of primers were used for the nested small subunit rRNA gene PCR. For the initial PCR, the forward E1 (5′-TTTGTATTAGTACAAA-3′) and reverse E2 (5′-GTA[A/G]TATTGATATACT-3′) primers which specified a 0.9-kb fragment for both *E. histolytica* and *E. dispar* were used. For the nested PCR, the forward Eh1 (5′-AATGGCCCATTCATTCAATG-3′) and reverse Eh2 (5′-TTTAGAAACAATGCTTCTCT-3′) primers, which specified only *E. histolytica*, were used. The initial and nested PCRs were performed as previously described (Haque *et al*., 1998).

Nested Real-Time PCR of SREHP gene With SYBR Green I

The DNA specimens, which were found positive for *E. histolytica* by nested ssrRNA gene PCR, were selected for the Real-Time PCR amplification of SREHP gene. The amplification was performed in two phases. In first phase, an initial PCR was performed, which was a conventional type and the forward SREHP-5 (5′-GCTAGTCCTGAAAAGCTTGAAGAAGCTG-3′) and reverse SREHP-3 (5′-GGACTTGATGCAGCATCAAG GT-3′) primers, which amplified a 549-bp fragment of SREHP gene of strain HMI:IMSS (Clark and Diamond, 1993) were used. For the second phase, a nested PCR was performed as Real-Time PCR using SYBR Green I. In second phase, the initial PCR product was used as template and here the forward nSREHP-5 (5′-TATTATTATCGTTATCTGAACTACTTCCTG-3′) and reverse nSREHP-3 (5′-TGAAGATAATGAAGATGA TGAAGATG-3′) primers, which amplified 450-bp fragment located within 549-bp of initial PCR product was used.

The initial PCR was conventional type and a hot start technique was performed. The PCR amplification was carried out in a total of 25 µl reaction volume. In case of stool/liver aspirate DNA, about 4 µl was mixed with 15.1 µl of autoclaved deionized H_2O and in case of cultured DNA, about 1 µl of DNA was mixed with 18.1 µl autoclaved deionized H_2O in 0.2 ml microcentrifuge tube. An amount of 5.9 µl of freshly prepared "mastermix" [which contained 2.5 µl of 10X PCR buffer, 1.8 µl of 50 mM $MgCl_2$, 0.3 µl of 25 mM dNTP mix, 0.3 µl of Taq polymerase (5 U/µl), 0.5 µl of 50 pmol forward primer SREHP-5 and 0.5 µl of 50 pmol reverse primer SREHP-3] was added to the tubes containing DNA and water and then overlaid with one drop of mineral oil (all reagents were obtained from Invitrogen^{TM}, USA). Then the tubes were taken in iCycler Thermal Cycler and the activation of *Taq* polymerase was done by a initial denaturation at 94ºC for 3 minutes and then 40 cycles of PCR were performed with denaturation at 94ºC for 60 sec, annealing at 45ºC for 60 sec and extension at 72ºC for 90 sec. After completion of 40 cycles, a final extension was done at 72ºC for 5 minutes and the product was maintained at 4ºC until the nested Real-Time PCR was done.

Real time PCR amplification was done by taking the initial PCR product of SREHP gene as a template. In This Real-Time PCR total reaction volume was of 25 µl. An amount of 1.0 µl of diluted initial PCR product (1:50) was added to 11.0 µl of deionized water in 0.2 ml well of experimental PCR plate. An amount of 13.0 µl of freshly prepared "mastermix" (which contained 12.5 µl of 2 X iQ SYBR Green Supermix, 0.25 µl of 50 pmol forward primer nSREHP-5 and 0.25 µl of 50 pmol reverse primer nSREHP-3) was added to the well containing water and DNA (the reagent for Real-Time PCR was obtained from Bio-Rad Laboratories, Inc.). The other steps were similar to the initial PCR of SREHP gene except the annealing temperature which raised from 45ºC to 55ºC. SYBR Green I is a double-stranded DNA binding dye and when it binds to double-stranded DNA, the fluorescence emission occurs which is used for the visualization of amplified product. During Real-Time PCR of SREHP gene with SYBR Green I, the amplification of each DNA specimen was determined by observing the fluorescence emission curves (Fig. 1). These curves were produced due to binding of SYBR Green I to the PCR product and the fluorescence reading were taken after each extension step of Real-Time PCR. The fluorescence emission increases due to the increase cycle of PCR.

Melting Curve Analysis

After completion of 40 cycles PCR amplification, the PCR products were melted by raising the temperature from 40ºC to 95ºC at a rate of 1ºC/min. The iCycler iQ software displayed the data collected during melt curve analysis as **–dF/dT vs Temperature** (Fig. 2). As a result melting temperatures were derived from melting peaks by melting curve analysis of the amplified DNA specimens. PCR products were analyzed on agarose gels to observe different types of banding patterns and to compare these patterns with melting temperatures. To this aim, products amplified on the iCycler Thermal Cycler were recovered from PCR plates, mixed with loading buffer, and loaded onto 1.3% agarose gels in 1×TBE (Tris-borate-EDTA) buffer. The gels were stained with ethidium bromide.

Statistical Analysis

Statistical analysis was done by the software ***"EP info"*** to compare the genotypes of *E. histolytica* between intestinal amoebiasis patients and amoebic liver amoebiasis patients

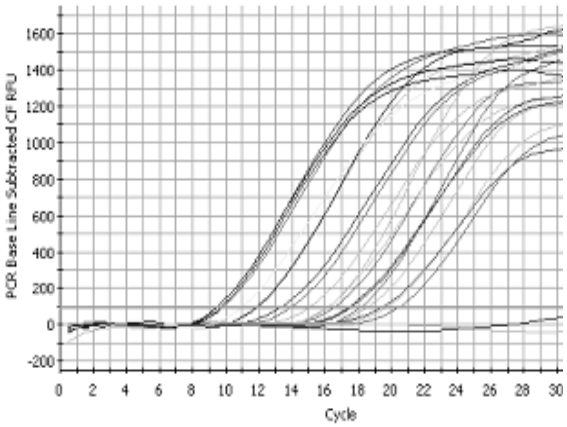

Fig. 1. Amplification of SREHP gene of E. histolytica with SYBR Green I using primers nSREHP-5 and nSREHP-3. *Amplification of the SREHP gene can be determined by observing the fluorescence emission curves. The fluorescence curves which exceed the threshold bar represent the amplified SREHP gene of E. histolytica. The DNA from nonamoebic stool did not amplified and the fluorescence curves remained below the threshold bar.*

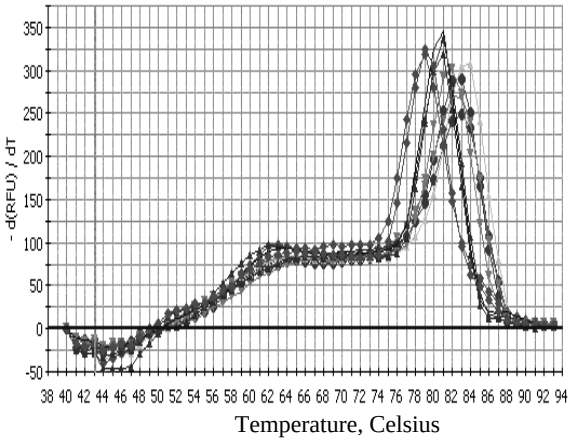

Fig. 2. Different types of melting peak of E. histolytica for SREHP gene amplification with SYBR Green I. *This graph represents the amplified DNA products as distinct melting peaks with specified melting temperatures. The melting temperatures are 84°C, 83°C, 82°C, 81°C and 79°C and each melting temperature is considered as a genotype.*

RESULTS AND DISCUSSION

Genotypes of E. histolytica

The SREHP gene was amplified from 60 *E. histolytica* isolates (collected from stool specimens of symptomatic and asymptomatic individuals and from liver aspirates of amoebic liver amoebiasis patients) by Real-Time PCR with SYBR Green I. The amplified products were then melted and by melting curve analysis five different melting temperatures e.g. 84°C, 83°C, 82°C, 81°C and 79°C were found (Fig. 2). Each melting temperature was considered as a genotype. As a result, five genotypes; genotype-I, genotype-II, genotype-III, genotype-IV and genotype-V were found for the melting temperature 84°C, 83°C, 82°C, 81°C and 79°C respectively (Table 1).

Table 1. Different melting temperatures of *E. histolytica* isolates from specimens of stool and liver abscess aspirates

Total number of isolates	Melting temperatures (Tm) °C	Genotypes	Number of isolates	Percentage of isolates (%)
60	84	Genotype-I	12	20
	83	Genotype-II	27	45
	82	Genotype-III	09	15
	81	Genotype-IV	08	13.33
	79	Genotype-V	04	06.67
Positive control (HM1: IMSS)	83	Genotype-II	01	–
Negative control (Non-amoebic stool)	Not amplified	–	02	–

The melting temperature for each peak is dependent on the GC/AT ratio, length, and nucleotide sequence of the PCR product (Ririe *et al*., 1997; Nicolas *et al*., 2002). Based on this principle, in this study we found 5 genotypes belonging to 5 different melting temperatures. Tanriverdi *et al*. (2002) for the genotyping of *C. parvum*, Amar *et al*. (2003) for the geotyping of *G. duodenalis*, Nicolas *et al*. (2002) for the differentiation of *Leishmania* species, Fujigaki *et al*. (2004) for the genotyping of Hepatitis C virus used Real-Time PCR with SYBR Green I and melting curve analysis. Their genotyping methods and results on the basis of melting curve analysis strongly support the present study for the genotyping of *E. histolytica* using Real-Time PCR and melting curve analysis. Among 60 *E. histolytica* isolates from specimens of stool and liver abscess aspirates, the genotype-II was found to be more prevalent (45%) than the genotype-I (20%), genotype-III (15%), genotype-IV (13.33%) and genotype-V (6.67%) (Table 1).

Genotypes of intestinal and amoebic liver amoebiasis patients

Among 50 *E. histolytica* isolates of intestinal amoebiasis patients, 20 (40%) isolates were to the genotype-II. On the other hand, among 10 *E. histolytica* isolates of amoebic liver amoebiasis patients, 7 (70%) isolates were to the genotype-II (Table 2). When the genotype-II was compared between the intestinal and amoebic liver amoebiasis patients, it was found that the genotype-II was more prevalent in amoebic liver amoebiasis patients, which was borderline significant (p = 0.09).

Table 2. Differences of genotypes among *E. histolytica* isolates of intestinal and amoebic liver amoebiasis patients

Genotypes of *E. histolytica*	Genotypes from stool isolates (n = 50)		Genotypes from liver abscess isolates (n = 10)	
	No.	%	No.	%
Genotype-I	12	24	0	0
Genotype-II	20	40	7	70
Genotype-III	08	16	1	10
Genotype-IV	07	14	1	10
Genotype-V	03	06	1	10

n = Number of stool isolates / liver abscess isolates.

Table 3. Genotypes of *E. histolytica* isolates from stool specimens of asymptomatic (nondiarrhoeal) and symptomatic (diarrhoeal) individuals

Genotypes of *E. histolytica*	Genotypes from asymptomatic (nondiarrhoeal) patients (n = 32)		Genotypes from symptomatic (diarrhoeal) patients (n = 18)	
	No.	%	No.	%
Genotype-I	06	18.75	6	33.33
Genotype-II	14	43.75	6	33.33
Genotype-III	05	15.62	3	16.67
Genotype-IV	06	18.75	1	05.56
Genotype-V	01	3.13	2	11.11

n = Number of stool isolates.

All the 5 genotypes were present in intestinal amoebiasis patients but in case of amoebic liver amoebiasis patients the genotype-I was absent which was statistically insignificant ($p > 0.05$), however we tested 10 isolates of *E. histolytica* from amoebic liver amoebiasis patients. Such interesting findings need to be studied further in future with more samples.

Out of 32 stool isolates of *E. histolytica* from asymptomatic individuals, 14 (43.75%) isolates were to the genotype-II and out of 18 isolates from diarrhoeal patients, 6 (33.33%) isolates were to the genotype-II. When the genotype-II from asymptomatic and symptomatic patients was compared with the genotype-II of amoebic liver amoebiasis patients, it was found insignificant ($p > 0.05$) (Table 3).

Comparison between the melting temperatures and nested SREHP PCR product (undigested) sizes for the genotyping of E. histolytica

For the genotyping of *E. histolytica*, a comparison was studied between the melting curve analysis method and the traditional PCR based typing method. For this, all the undigested nested SREHP PCR products belonging to five melting temperatures were run by agarose gel electrophoresis (Fig. 3) and as a result 23 different banding patterns were found (Table 4) which indicated extensive genetic variation among *E. histolytica* isolates.

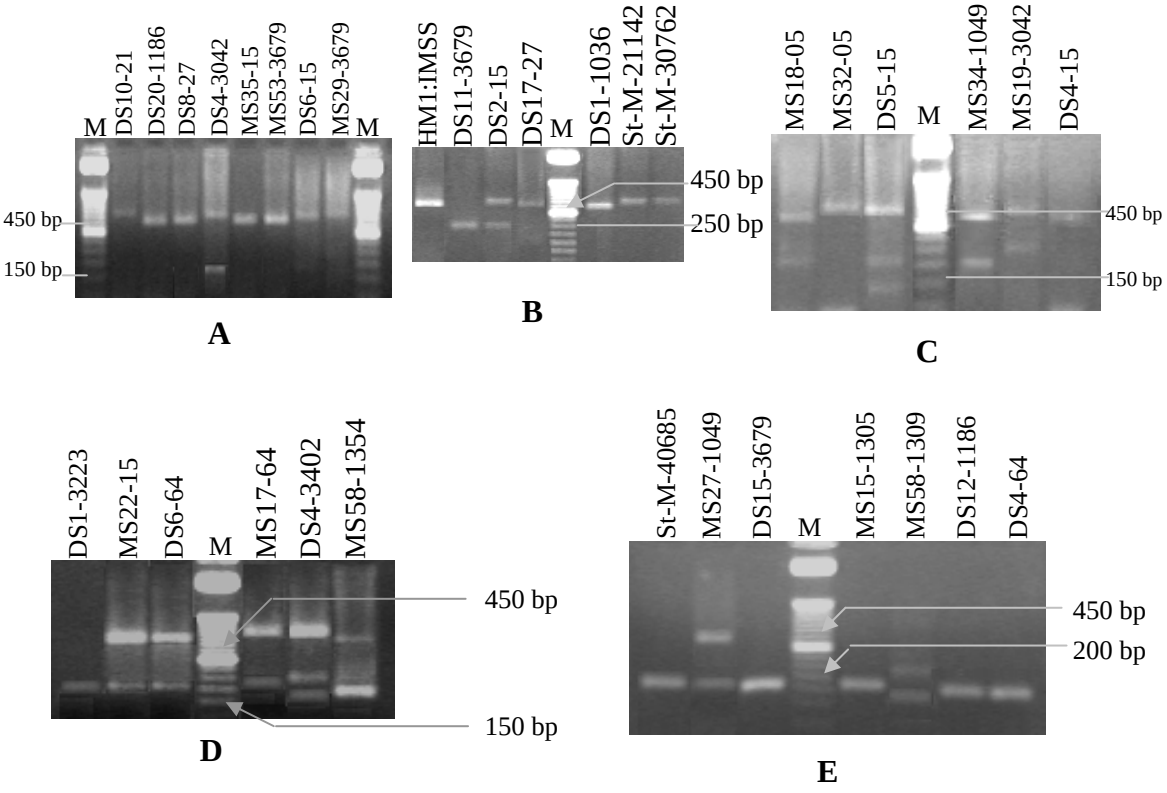

Fig. 3. ***Gel photograph of nested SREHP PCR products (undigested) from E. histolytica isolates belonging to the melting temperature 84°C (A), 83°C (B & C), 82°C (D), 81°C and 79°C (E).*** *After the Real-Time PCR amplification of SREHP gene and melting curve analysis, the PCR products belonging to the different melting temperatures were run in a 1.3% agarose gel. Isolate designations are shown at the top. In gel photograph B, HM1: IMSS strain represents the control DNA amplification from culture. Lane M represents 50 base pair DNA marker (Invitrogen®).*

Ghosh *et al*. (2000), Zaki and Clark (2001), Ayeh-Kumi *et al*. (2001) and Haghighi *et al*. (2002) also found extensive genetic variations among *E. histolytica* isolates by using traditional PCR based methods. But in this study, we found 5 distinct genotypes using Real-Time PCR and melting curve analysis.

Table 4. Comparison between the melting temperatures and nested SREHP PCR product (undigested) sizes

Genotypes	Melting temperature (°C)	Nested SREHP- PCR product Size (bp)	Pattern No.	Frequency of band pattern
Genotype-V	79	120	1	1
		140	2	1
		170	3	2
Genotype-IV	81	180	4	3
		190	5	3
		240, 140	6	1
		430, 180	7	1
Genotype-III	82	450, 200	8	5
		450+, 210	9	1
		240	10	1
		200	11	1
		450+, 240, 180	12	1
Genotype-II	83	410	13	2
		440	14	1
		450	15	14
		410, 230	16	3
		450, 260	17	3
		450, 230, 130	18	1
		400	19	1
		450, 220, 190	20	1
		250	21	1
Genotype-I	84	450+	22	11
		450+, 160	23	1

Moreover, the traditional PCR based typing techniques require more time, intensive and laborious post-PCR manipulations (like agarose gel electrophoresis and RFLP analysis), make use of hazardous chemicals, and carry a potential risk for laboratory contamination. On the other hand, this Real-Time PCR assay has a considerable advantage over the previously reported assay because of the speed of analysis and excluding the post PCR manipulation. Thus this Real-Time PCR assay with SYBR Green I and melting curve analysis would be helpful for the rapid screening of genotypes of *E. histolytica* among endemic population and also for the epidemiological studies. This method of genotyping of *E. histolytica* by Real-Time PCR with SYBR Green I and melting curve analysis should be compared in future with the other method of Real-Time PCR with different probes and dye to observe different genotypic variations. Each genotype (for each melting temperature) found in this study should be sequenced in future as within each genotype different banding pattern of PCR products were found.

REFERENCES

1. Amar CFL, Dear PH and McLauchlin J (2003). Detection and genotyping by real-time PCR/FRLP analyses of *Giardia duodenalis*. *Journal of Medical Microbiology* 52: 681-683.
2. Ayeh-Kumi PF, Ali IKM, Lockhart LA, Gilchrist CA, Petri WAJr and Haque R (2001). *Entamoeba histolytica*: Genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the Serine-Rich Gene. *Experimental Parasitology* 99: 80-88.
3. Bhattacharya S, Bhattacharya A and Diamond LS (1992). *Entamoeba histolytica* extrachromosomal circular ribosomal DNA: analysis of clonal variation in a hypervariable region. *Experimental Parasitology* 74: 200-204.
4. Burch DJ, Li E, Reed S, Jackson TFHG and Stanley SLJr (1991). Isolation of a strain –specific *Entamoeba histolytica* cDNA clone. *Journal of Clinical Microbiology* 29: 696-701.
5. Clark CG and Diamond LS (1993). *Entamoeba histolytica*: a method for isolate identification. *Experimental Parasitology* 77: 450-455.
6. Fujigaki H, Takemura M, Takahashi K, Yamada Y, Fujii H, Wada H, Saito K, Ohnishi H and Seishima M (2004). Genotyping of hepatitis C virus by melting curve analysis with SYBR Green I. *Annals of Clinical Biochemistry* 41 (Pt 2): 130-132.
7. Ghosh S, Frisardi M, Ramirez AL, Descoteaux S, Sturm RK, Newton SOA, Santos PJI, Ganguly C, Lohia A, Reed S and Samuelson J (2000). Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (Demographic sweep) and transcontinental spread of diploid parasites. *Journal of Clinical Microbiology* 38: 3815-3821.
8. Haque R, Faruque ASG, Hahn P, Lyerly DM and Petri WAJr (1997). *Entamoeba histolytica* and *Entamoeba dispar* infection in children in Bangladesh. *Journal of Infectious Diseases* 175: 734-736.
9. Haque R, Ali IKM, Akther S and Petri WAJr (1998). Comparison of PCR, isoenzyme analysis and antigen detection for diagnosis of *Entamoeba histolytica* infection. *Journal of Clinical Microbiology* 36: 449-452.
10. Haque R, Ali IKM and Petri WAJr (1999). Prevalence and immune response of *Entamoeba histolytica* infection in preschool children in Bangladesh. *American Journal of Tropical Medicine and Hygiene* 60: 1031-1034.
11. Haghighi A, Kobayashi S, Takeuchi T, Masuda G and Nozaki T (2002). Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. *Journal of Clinical Microbiology* 40: 4081-4090.
12. Jackson TFHG, Gathiram V and Simjee AE (1985). Seroepidemiological study of antibody responses to the zymodemes of *Entamoeba histolytica*. *Lancet* 1: 716-719.
13. Kohler S and Tannich E (1993). A family of transcripts (K_2) of *Entamoeba histolytica* contains polymorphic repetitive regions with highly conserved elements. *Molecular and Biochemical Parasitology* 59: 49-58.
14. Li E, Kunz-Jenkins C and Stanley SLJr (1992). Isolation and characterization of genomic clones encoding a serine-rich *Entamoeba histolytica* protein. *Molecular and Biochemical Parasitology* 50: 355-357.
15. Nicolas L, Milon G and Prina E (2002). Rapid differentiation of Old World *Leishmania* species by LightCycler polymerase chain reaction and melting curve analysis. *Journal of Microbiological Methods* 51: 295– 299.
16. Ravdin JI (1995). Amoebiasis. State-of-the-art clinical article. *Clinical Infectious Diseases* 20: 1453-1466.
17. Ririe KM, Rasmussen RP and Wittwer CT (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* 245: 154-160.
18. Sargeaunt PG, Williams JE and Grene JD (1978). The differentiation of invasive and non-invasive *Entamoeba histolytica* by isoenzyme electrophoresis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 72: 519-521.
19. Sargeaunt PG (1988). Zymodemes of *Entamoeba histolytica*. In "*Amebiasis: Human Infection by Entamoeba histolytica*" (Ravdin, J. Ed.), pp 370-387, Wiley, New York.
20. Sehgal D, Bhattacharya A and Bhattacharya S (1993). Analysis of a polymorphic locus present upstream of rDNA transcription units in the extrachromosomal circle of *Entamoeba histolytica*. *Molecular and Biochemical Parasitology* 62: 129-130.
21. Stanley SLJr, Becker A, Kunz-Jenkins C, Foster L and Li E (1990). Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. *Proceedings of the National Academy of Sciences of the United States of America* 87: 4976-80.
22. Tanriverdi S, Tanyeli A, Baslamish F, Koksal F, Kihnc Y, Feng X, Batzer G, Tzipori S and Widmer G (2002). Detection and genotyping of oocysts of *Cryptosporidium parvum* by Real-Time PCR and Melting Curve Analysis. *Journal of Clinical Microbiology* 40: 3237-3244.
23. Zaki M and Clark CG (2001). Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. *Journal of Clinical Microbiology* 39: 897-905.