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Genetic variability of the Serine-Rich *Entamoeba histolytica* protein gene in clinical isolates from the United Arab Emirates

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Abstract. The genetic diversity of 20 *Entamoeba histolytica* isolates from asymptomatic individuals from the UAE was investigated by analyzing polymorphism in the serine-rich *E. histolytica* gene (SREHP) by nested polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (RFLP) on DNA extracted directly from stool samples. The SREHP gene was successfully amplified in 15 out of 20 *E. histolytica*-positive samples. Four out of the remaining five isolates did not amplify for the SREHP gene. Despite successful amplification of the SREHP gene in the fifth isolate, *Alu*I digestion of the amplified PCR product revealed no bands. As a result, all five samples were excluded from the study. Twelve different profiles were obtained from the 15 successfully amplified isolates. Thus, demonstrating extensive genetic variability and reinforcing the argument that *E. histolytica* has an extremely polymorphic genetic structure. Despite the sample size limitation, a finding in the study was the occurrence of one profile common to one Indian isolate while another profile common to one Pakistani isolate; indicating the possibility of clonal infection. Furthermore, we found one isolate from a Bangladeshi expatriate identical to 2 asymptomatic Bangladeshi isolates reported in an earlier study. No clear association between the different genotypes and the study population demographics was noted. The results also indicated the possibility of strains clustering by region.

INTRODUCTION

Amoebiasis, a substantial health problem in many developing and tropical regions of the globe, is a parasitic disease caused by *Entamoeba histolytica*. The disease manifestations which include invasive intestinal and extra-intestinal amoebiasis, cause up to 100,000 deaths worldwide annually (Petri & Singh, 1999). Currently, the determinants of the disease are not well understood as 90% of infected individuals are usually asymptomatic and roughly 10% may develop intestinal and/or extra-intestinal diseases such as diarrhea, colitis and amoebic liver abscess (Jackson *et al.*, 1985; Haque *et al.*, 1997). This observation might partly be due to the differences in the genetic composition and pathogenic potential of infecting strains (Burch *et al.*, 1991; Ali *et al.*, 2007) as well as the host immune backgrounds (Mortimer & Chadee, 2010) and diet (Stanley, 2003).

In 1993, Clark and Diamond established evidence of the existence of inter-strain variations in *E. histolytica*; their studies on *E. histolytica* cultures from different geographical areas of the world confirmed the presence of a wide degree of polymorphism in two *E. histolytica* genes, the "serine-rich protein" (SREHP) and the "strain-specific gene" (SSG) (Clark & Diamond,

1993). Each of which has internal tandem repeat sequences. Size and restriction site polymorphisms identified in the repetitive genes yielded 16 distinct DNA patterns of 18 axenically cultured *E. histolytica* isolates. The polymorphism of *E. histolytica* strains within and between endemic areas has been demonstrated recently in different countries (Ghosh *et al.*, 2000; Ayeh-Kumi *et al.*, 2001; Haghighi *et al.*, 2003; Samie *et al.*, 2008). For example, Ayeh-Kumi *et al.* (2001) detected 34 dissimilar SREHP profiles among 54 isolates from children in the Mirpur region of Dhaka, Bangladesh. In the same study, an existence of some degree of association between the SREHP genotype and disease outcome was indicated.

In general, little is known about the extent of intestinal parasitic infections in inhabitants of countries in the Middle East, especially those in the Arabian Peninsula, nevertheless some reports have been published (Astal, 2004; Idris & Shaban, 1992). We previously studied the incidence rate of *E. histolytica* in Sharjah, UAE, using nested PCR where we reported an average incidence rate of 13.3% (ElBakri *et al.*, 2013). However, larger studies need to be conducted in order to confirm these findings. Among the many genetic loci used to investigate the population structure of *E. histolytica* isolates, the SREHP locus is one important marker widely used in strain typing and virulence studies. This gene has shown wide diversity in past studies in other regions (Haghighi *et al.*, 2003; Ayeh-Kumi *et al.*, 2001). Information on intra-species variation within *E. histolytica* is much needed to accurately investigate the epidemiology of the organism in endemic areas. To date, no report on the sub-typing of *E. histolytica* strains in the UAE is available. Therefore, in the present study we aimed to identify the genetic diversity of *E. histolytica* strains based on the polymorphism of the SREHP gene locus using a nested PCR-RFLP as previously described (Ayeh-Kumi *et al.*, 2001) in Sharjah, UAE, which is not an endemic area.

MATERIALS AND METHODS

Stool specimens, DNA extraction and PCR detection of *E. histolytica*

The complete description of the study samples, extraction method and nested PCR method used has been presented previously (ElBakri *et al.*, 2013). Briefly, one hundred and thirty four fresh unpreserved fecal samples screened microscopically for *E. histolytica /E. dispar/ E. moshkovskii* complex trophozoites and/or cysts were collected in sterile capped containers and transported without delay to the University of Sharjah. All specimens were collected from asymptomatic migrant workers presenting to the Sharjah Municipality Public Health Clinic (SMPHC) for intestinal parasites screening. Genomic DNA was extracted from all stool samples using the QIAamp stool DNA Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's recommendations. The nested PCR conditions were performed as described by Khairnar & Parija (2007), with slight modifications. All samples positive for *E. histolytica* including those with mixed infections were subjected to SREHP gene amplification.

Nested PCR amplification of serine rich *Entamoeba histolytica* protein gene from stool samples

Entamoeba histolytica HM-1: IMSS, originally isolated from a patient with colitis in 1967 was used as a positive control in this study. The genetic diversity of *E. histolytica* isolates and positive stools was determined through the polymorphism of the serine-rich *E. histolytica* protein (SREHP). The protocol described by Ayeh-Kumi *et al.* (2001) was used. Briefly, a set of primers (SREHP5 and SREHP3) which amplify a 549-bp fragment of the SREHP gene of strain HM-1:IMSS were used for the initial PCR, followed by a second set of primers (nSREHP5 and nSREHP3) located within the fragment amplified for the nested PCR and resulting in a 450-bp fragment for the axenic isolate HM-1:IMSS.

In the first PCR, 5 µl of DNA was added to 20 µl of a master mix composed of 0.6 µl each of 50 pmol solution of primers (SREHP5 and SREHP3), 2.5 µl of 10 x PCR buffer, 0.6 µl of 10 mM dNTP mix, 1.8 ml of 25 mM $MgCl_2$, and 0.4 µl of Taq Polymerase (Qiagen, Germany). After an initial step of the Taq Polymerase activation at 95ºC for 15 min, 40 cycles with denaturation at 94ºC for 1 min, annealing at 50ºC for 1.5 min, and extension at 72ºC for 2 min were performed in a Master cycler gradient (Eppendorf; Hamburg, Germany). This step was followed by the nested PCR using 1 µl of the initial PCR product as the template DNA. The annealing temperature was raised from 50 to 55ºC for the nested PCR while the other parameters of the amplification cycle remained unchanged. The sequences of the primers were: SREHP5–5' GCT AGT CCT GAA AAG CTT GAA GAA GCT G 3', SREHP3–5' GGA CTT GAT GCA GCA TCA AGG T 3', nSREHP5–5' TAT TAT TAT CGT TAT CTG AAC TAC TTC CTG 3', nSREHP3–5' TGA AGA TAA TGA AGA TGA TGA AGA TG 3'. DNA isolated from axenic cultures of *E. histolytica* HM-1: IMSS was used as a positive control for PCR amplifications. Stool specimens that were negative by microscopy and the 16S-like ribosomal RNA gene (ElBakri *et al.*, 2013) were used as negative controls. Fragment sizes were estimated using the 100-bp ladder (Promega, USA).

*Alu*I digestion of the nested SREHP PCR products

The PCR products were additionally analyzed following digestion with the restriction endonuclease *Alu*I (Promega, USA); 10 µl of PCR products were digested for 2 h at 37ºC using the manufacturer's recommended procedure. The PCR and the digested products were separated electrophoretically in 1.3% and 2.1% agarose gels (respectively). Agarose gels were stained with ethidium bromide and were visualized by UV light. Agarose gels were documented by photography, and fragment sizes were estimated using the 100-bp DNA ladder (Promega, USA).

RESULTS

Of the 20 samples positive for *E. histolytica* (including 4 samples co-infected with *E. dispar*), 14 (70%) were males and 6 (30%) were females. The nested SREHP PCR and *Alu*I RFLP analyses were used to investigate the genetic variations among the 20 isolates of *E. histolytica* collected from asymptomatic individuals in Sharjah, UAE. The SREHP gene was successfully amplified in 15 out of all 20 *E. histolytica*-positive samples. Four out of the remaining five isolates did not amplify for the SREHP gene. However, despite successful amplification of the SREHP gene in the last isolate (isolate #30), *Alu*I digestion of the amplified PCR revealed no bands. As a result, all five samples were excluded from the study. The nested SREHP PCR reaction and the *Alu*I digestion gave different banding patterns as represented in Figure 1 (A, B, and C), respectively. Figure 1 (B) shows a representation of an isolate (#105) which did not amplify for the SREHP gene. Furthermore, due to technical difficulties and the degradation of the amplified PCR products we could not obtain the sequences for all the isolates. Both size and restriction site polymorphisms were detected among the 15 isolates. The major band sizes and profiles obtained from the stool samples are presented in (Table 1). For the nested PCR products, the band sizes varied between 150 bp and 700 bp while the size of the digested products varied between 70 bp and 400 bp (Table 1). The nested PCR amplification of genomic DNA from *E. histolytica* HM-1: IMSS strain as a positive control resulted in the amplification of the expected 550 and 450 bp bands, respectively. The nested SREHP PCR amplification resulted in the appearance of the characteristic 450 bp in 7 out of 15 DNA samples that were successfully amplified, with additional lighter 200bp bands found in most samples. Other bands 300-350 and 500 were also seen in most samples (Fig. 1 A and B; Table 1). Greater polymorphism of the SREHP gene was observed between the different isolates after digesting the

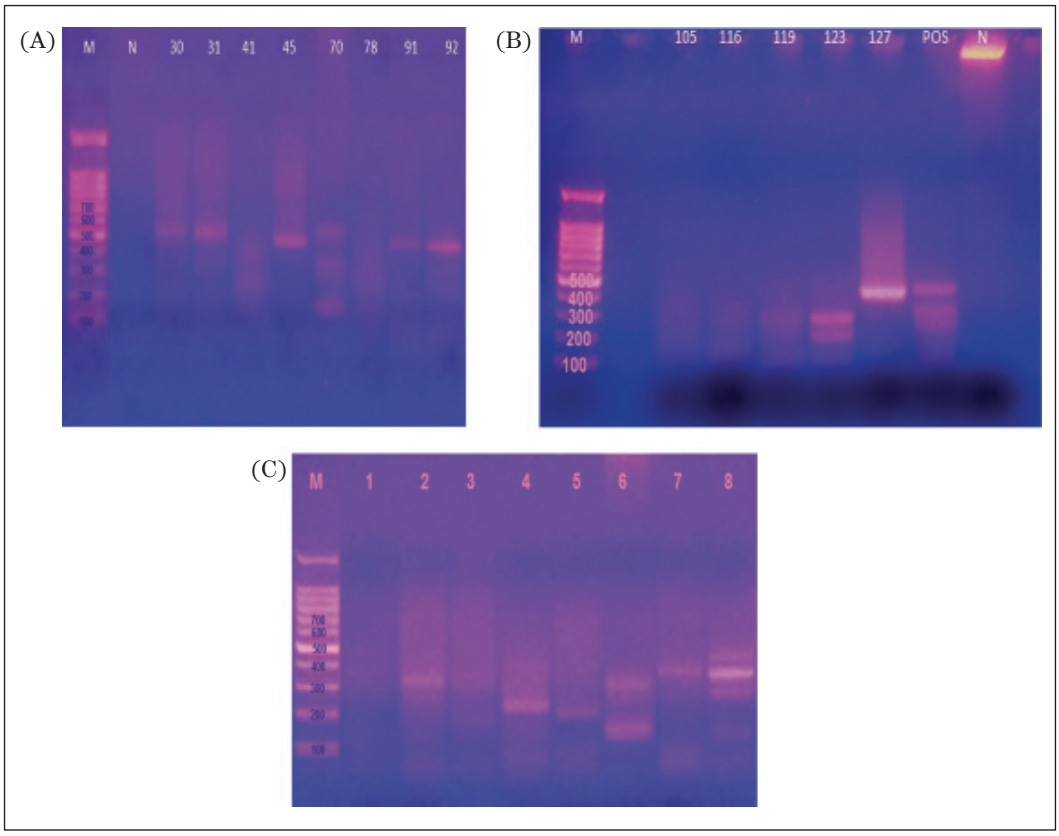

Figure 1. Nested SREHP PCR products from selected isolates (A and B) and their *Alu*I digested profiles (C). (A) and (B): Lane M, DNA size marker, 100 bp DNA ladder (Qiagen). Lane Pos, positive control HM-1: IMSS. Lane N, negative control (without template DNA). C. *Alu*I-digested nested SREHP PCR products from the selected isolates. Nested SREHP PCR products of (A) were digested with restriction enzyme *Alu*I and the fragments were separated in a 2.1% agarose gel. Lanes 1–8 represent test isolates: #1-30; #2-31; #3-41; #4-45; #5-70; #6-78; #7-91; and #8-92. Lane M, DNA ladder, 100 bp DNA ladder (Qiagen). The major bands are represented in the tables

nSREHP products with the *Alu*I restriction enzyme. *Alu*I digestion of the PCR products revealed 12 different profiles (Fig. 1 C) (Table 1). Seven isolates were from the Indian subcontinent (5 males and 2 females), and based on the *Alu*I digestion of the PCR products, all except two isolates generated different profiles (profile # 4). Another profile observed in two samples was profile #7. Interestingly, both isolates were from two male Pakistani national expatriates of similar age in the UAE. Whether this is just a coincidence or they may possibly have been infected with the same strain is difficult to establish given that the sample size studied was small. Moreover, one Indian isolate shared the same profile as an Indonesian isolate (profile # 1). Three different profiles were also observed in isolates for the Philippines, Bangladesh, Sri Lanka, Afghanistan and Nepal.

DISCUSSION

Using the polymorphic SREHP gene (Ayeh-Kumi *et al.*, 2001), we sought to determine the diversity of *E. histolytica* strains in the study sample in Sharjah, UAE. The SREHP gene successfully amplified in fifteen isolates out of the 20 *E. histolytica* positive samples included in the study. Four isolates did not

Table 1. Genetic diversity of *E. histolytica* in stool samples from Sharjah, UAE

Isolate No.	Nationality	Sex	Age	nSREHP	AluI digestion	Profile No.	Freq.
4	India	M	20	150, 300	270	1	2
11	India	M	43	–	–	–	–
16	India	M	25	320	170[d], 270	2	1
23	India	M	28	–	–	–	–
30	India	M	33	240,450, 500	–	–	–
41	India	F	24	200[f], 270, 450	70, 210, 300	3	1
45	India	M	27	450	70-90, 250, 350	4	2
78	India	M	41	200[f], 270, 450	70-90[f], 150[d], 170, 270, 300	5	1
116	India	M	48	200-250[f]	120, 200, 320	6	1
91	India	F	26	450	70-90, 250, 350	4	2
21	Indonesia	F	44	180, 500	270	1	2
22	Pakistan	M	21	200, 300	200[f], 300[f]	7	2
123	Pakistan	M	29	200, 280-300	200, 300	7	2
119	Bangladesh	M	22	280-320	90-100	8	1
127	Sri Lanka	F	41	450	80-100, 200	9	1
31	Philippines	F	25	350, 450, 500	70, 100, 250, 300	10	1
70	Afghan	M	32	170, 350, 560	70, 100, 200	11	1
85	Egypt	M	45	–	–	–	–
92	Nepal	M	29	450, 700[f]	70, 150, 270, 300, 400	12	1
105	Iraq	F	47	–	–	–	–
	Negative Control			Negative			
	Pos. HM1 : IMSS			320, 450	170	–	–

Notes: superscript [f]Faint, [d]doublet. Major bands obtained after the nested PCR of the SREHP (nSREHP) gene and after digestion with *Alu*I. Freq is the frequency of that specific genetic pattern in the samples population

amplify for the SREHP gene (isolate numbers 11, 23, 45, and 105). Although, isolate 30 successfully amplified for the 450 bp SREHP gene, its *Alu*I digestion resulted in no bands. As a result, they were excluded from the study. Interestingly, the 15 isolates in the study exhibited considerable polymorphism in the SREHP gene and the comparison of isolates from individuals from different geographical regions of origin was of note. The band sizes of the *Alu*I digested product obtained in the present study were also in the range found earlier by other authors (Ayeh-Kumi *et al.*, 2001; Haghighi *et al.*, 2003; Simonishvili *et al.*, 2005; Samie *et al.*, 2008; Tanyuksel *et al.*, 2008). Nonetheless, the profiles were slightly different from those obtained in previous studies indicating a wide variability of the SREHP gene in different geographic areas of the world. Twelve different profiles were obtained in the present investigation (Table 1). To our surprise, one Bangladeshi strain (isolate no. 119) showed a SREHP genotype identical to 2 asymptomatic Bangladeshi isolates from Ayeh-Kumi *et al.* (2001) study. Another finding in the study was the occurrence of four identical isolates having the same profile numbers (from Pakistan, isolates 22 and 123; from India, isolates 45 and 91). All four isolates were from individuals of similar age group (refer to Table 1).

The results obtained in the present study indicated that there was a possibility of the existence of the same strain infecting individuals from the same region. Moreover, it also showed that there is a possibility of strains clustering by region. This finding corroborated previous findings in a study carried out by Fu *et al.* (2010), in which it

was concluded that all isolates from different mental institutions in Japan were derived from a single source of *E. histolytica* source. Although the sample size in the present study was small to allow us to draw definite conclusions, we still were able to obtain higher diversity than those reported by others in earlier studies. Rivera *et al*. (2006) described six different profiles from among 74 positive isolates in a study in the Philippines whereas 22 profiles from a total of 79 positive isolates were reported by Haghighi *et al*. (2003). Yet, our results were comparable to those obtained by Samie *et al*. (2008) (26 profiles from 38 isolates), Ayeh-Kumi *et al*. (2001) (25 profiles from 42 positive stool isolates, and Tanyuksel *et al*. (2008) who reported 12 dissimilar profiles from 26 isolates. After calculating the ratio of the number of isolates by the number of profiles in all of the abovementioned studies, the following values were obtained respectively 12.3, 3.6, 1.4, 1.6 and 2. On the other hand, our study resulted in a ratio of 1.25 (15/12) making it the study with the highest diversity of all studies conducted so far. This is justified by the diverse geographical origin of the isolates. Earlier studies have suggested a possible correlation between strain genotype and pathogenic characteristics (Ayeh-Kumi *et al*., 2001; Simonishvilli *et al*., 2005; Samie *et al*., 2008); however, in the present study we were not able to test this hypothesis as all isolates were from asymptomatic individuals.

Few potential limitations of this study require comment: (1) all samples were from healthy expatriates presenting themselves to the SMHC for work and residency permits health checks. It was not possible to obtain stool samples from neither Emirati natives nor symptomatic patients; (2) Since we could not analyze cultured samples, most of our original DNA samples and/or amplified PCR products were either of poor quality or have degraded and therefore we could not obtain sequences of our test samples although Fu *et al*. (2010) recommended that when DNAs from xenic cultures or fecal samples are used for PCR amplification, comparison by sequencing is recommended because *Alu*1 recognition sites is also present in the sequence of the fragment from *V. parvula*, part of the bacterial flora of the human intestinal tract. We were not able to retest them because the original stool samples or DNA isolated from the stools are currently unavailable.

Together with past studies, it reinforces the argument that *E. histolytica* has an extremely complex genetic structure independent of geographic location. We have shown that *E. histolytica* is seen among the expatriate population and since the demographics of the UAE show that there is a large population of maids/drivers, helpers and food handlers from the Indian subcontinent, Southeast Asia and Africa where poor socio-economic levels and inadequate medical care are common (WHO, 1987), they may harbor the parasite and thus may be a source of indigenous transmission. The possible spread of the different strains of *E. histolytica* right through the native Emirati local community and other groups of migrants also needs to be considered, in the context of these working groups. On the other hand, there is an urgent need for further studies within the native Emirati population before any conclusion can be made about expatriates transmitting *E. histolytica* to the local community. A survey of prevalence of intestinal parasites among the expatriates and native Emirati people attending Ministry of Health hospitals in Sharjah, United Arab Emirate was performed during the year 2008 and 2009 where stool samples from 10,514 patients (64% expatriates and 36% native Emiratis) were examined. Eight hundred fourteen specimens of the 10,514 examined were found to be positive for intestinal parasites. The infection rates were 15.7% and 3.2% among the native and expatriate population respectively (odds ratio = 5.5). The rate of infection in males (58%) was higher than in females (42%). Overall, protozoa infections (92.2%) were higher than the helminth infections (7.8%). *Entamoeba histolytica/E. dispar* (71.8%) and *G. lamblia* (17.5%) were the commonest intestinal parasites identified. In comparison to worm infections, protozoa infections were more common among the native Emirati population than the expatriate population. The

high prevalence rate of intestinal parasitic infections among the local Emirati people clearly indicates that there is continuous ongoing transmission of various parasitic infections in the community (Dash *et al.*, 2010). These observations therefore showed the promise of SREHP in providing better understanding of the epidemiology and transmission mechanisms of *E. histolytica* infections in the country.

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