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Cloning and Characterization of Serine-rich *Entamoeba histolytica* Protein Gene from an Iranian *E. histolytica* Isolate

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Abstract: *Entamoeba histolytica* causes amebic dysentery and amebic liver abscess, placing it second only to malaria, as a cause of death resulting from parasitic protozoa. Serine-rich *Entamoeba histolytica* Protein gene is an immunogenic, polymorphic protein and a candidate of vaccine that was cloned and characterized in this research. An Iranian *Entamoeba histolytica/Entamoeba dispar* isolate was cultivated in Robinson Medium. Total genomic DNA was isolated and *Entamoeba histolytica* strain has been distinguished by PCR with two sets of species-specific primers and SREHP gene was amplified by Nested-PCR. The purified PCR product was cloned into pBluscript via T/A cloning method. The recombinant plasmid was screened by Rusconis test, digested with BamH1 restriction enzyme, which was approved by direct PCR and sequencing. The digested fragment of SREHP gene from recombinant plasmid was sequenced and showed a 666 base pair nucleotides as a new genotype of SREHP *Entamoeba histolytica*. The SREHP gene from Iranian isolate (SHR 10 IR) was different from HM1-IMSS cl.6 as well as all previously reported *Entamoeba histolytica* genotypes. Isolation of SREHP, expression of the gene, makes an opportunity to develop diagnostic kit, as well as determination of genetic variation of *Entamoeba histolytica* isolates in Iran.

Key words: Cloning, characterization, SREHP, *Entamoeba histolytica*, Iran

INTRODUCTION

The protozoan pathogen *Entamoeba histolytica* is the causative agent of invasive intestinal and extraintestinal amoebiasis. It is estimated that more than 10% of people are infected with *Entamoeba dispar* and *E. histolytica* strains worldwide annually. Current WHO estimates of 40-50 million cases of amebic colitis and liver abscess and up to 100000 deaths per annum, placing it second only to malaria in mortality due to this protozoan parasite (Stanley, 2003; Ravdin and Stauffer, 2005; WHO/PAHO/UNESCO, 1997). *E. histolytica/E. dispar* is distributed through the world and is a substantial health risk in almost all worlds especially developing countries (Stanley, 2003). Serine rich *Entamoeba histolytica* Protein (SREHP) is highly immunogenic and appears to possess a number of conserved epitope. Among individuals with amebic liver abscess more than 80% have antibodies that recognize the SREHP molecule (Stanley, 1997). There are different *E. histolytica* proteins that have formed the basis of recombinant antigen vaccines i.e., the SREHP, Gal/Gal NAC binding lectin, cystein-proteinase and a 29 KDa antigen (Stanley, 1997; Martinez-Palomo *et al*., 1998). Recombinant SREHP could be used in diagnosis of acute invasive amoebiasis (Myung *et al*., 1992). Considering WHO recommendation on necessity of molecular studies on *E. histolytica* specific immunogenic compound for vaccine development, this research was done to clone and characterize of SREHP gene, for future expression of it and preparation recombinant protein to make diagnostic kit and amoebiasis vaccine study.

MATERIALS AND METHODS

This research was conducted in Department of Parasitology and Mycology, School of Medicine,

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***Entamoeba histolytica* isolation:** An *E. histolytica/ E. dispar* strain was isolated from a dysenteric patient in Shiraz, Fars Province, Iran in 2002 year. Xenic *in vitro* culture was established and maintained in Robinson medium as previously described (Robinson, 1968). Axenized *E. histolytica* HM-1: 1 MSS cl.6 strain was used as control and maintained in TYI-S-33 medium (Diamond *et al*., 1978).

DNA preparation and PCR analysis: Total genomic DNA was isolated from culture as previously described (McPherson and Moller, 2000; Sambrook *et al*., 2001). *E. histolytica* strain has been distinguished by PCR, using two sets of species- specific primers from locus 1-2, i.e., HSP1+HSP2 and DSP1+DSP2, (Table 1) (Zaki *et al*., 2002). PCR was carried out in a 25 μL reaction mixture containing 200 ng of DNA, 1.5 μM concentration of primers, 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphate (dNTP) and 0.2 μL of Taq DNA Polymerase (CinnaGen inc, Iran) with the following cycling parameters: (i) Taq activities at 94°C for 2 min; (ii) 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 60 sec and (iii) extension at 72°C for 10 min. PCR products were electrophoresed in 1.5% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, Maine). The result was visualized after staining with ethidium bromide (Sambrook *et al*., 2001) in a UV transilluminator (UVIdoc Deluxe GAS 9000, England).

Preparation of DNA insert: External and internal primers of SREHP gene were designed based on SREHP gene obtained from HM-1: IMSS strain in GenBank database with accession no: M80910 and BamH1 site was designed at 5'end of internal primers (Table 1). A fragment of SREHP gene with about 700 bp was amplified using those primers by Nested-PCR. External PCR was carried out in a 50 μL reaction mixture containing 200 ng of DNA, 2 μM concentration of external primers, 1.5 mM $MgCl_2$, 0.2 mM of dNTP and 0.3 μL of Taq DNA Polymerase with the following cycling parameters: (I) Taq activities at 94°C for 2 min; (ii) 30 cycles of denaturing at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 90 sec and (iii) extension at 72°C for 10 min. Internal PCR carried out in a 50 μL reaction mixture containing 1 μL of first PCR product, 2 μM concentration of internal primers 1.5 mM $MgCl_2$, 0.2 mM of dNTP and 0.3 μL of Taq DNA Polymerase in the same external cycling parameters with the exception of annealing at 60°C for 30 sec. PCR product was purified by Q-Biogen Gene clean II Kit (BIO101, Lajolla, Calif).

Table 1: Oligonucleotide primers

Primer name	Primer sequence 5` to 3`
HSP1 (Forward)	GAGTTCTCTTTTTATACTTTTATATGTT
HSP2 (Reverse)	ATTAACAATAAAGAGGGAGGT
DSP1 (Forward)	TTGAAGAGTTCACTTTTTATACTATA
DSP2 (Reverse)	TAACAATAAAGGGGAGGG
EXT-SREHP (Sense)	GAGTGGAACATAATATCGATGA
EXT-SREHP (Antisense)	ATCCCAACTAAAGACTTTATT
SREHP-BamH1 (Sense)	GAGGATCCATGTTCGCATTTTTATTGT
SREHP-BamH1 (Antisense)	GAGGATCCTTAGAAGATGATAGCTAT

T vector preparation: pBluscript (pBSc) was extracted by GenElute™ plasmid mini prep kit (SIGMA, PLN-70) and T vector was prepared as previously described (Sambrook *et al*., 2001).

Gene cloning: The PCR product of SREHP gene was cloned into the T- pBluscript -cloning vector. T/A cloning was done in 20 μL volume, using insert-vector ratio of 4/1 with addition of 2 μL T4 DNA ligase. The reaction incubated at 22° C for 3 h and was transformed into DH5a competent cells (Sambrook *et al*., 2001). Recombinant plasmid was sequenced with appropriate internal primers in both directions using System analysis DNA Sequence 2000XL Beckman coulter, USA. The sequences obtained were manually edited and aligned using GENERUNR (Version 3.05).

Screening: Recombinant plasmids were detected by blue/white colonies screening method (Sambrook *et al*., 2001) and was confirmed by Rusconis test. For the rusconis test a white colony was picked up and mixed with 12 μL rusconis solution, centrifuged at 12000 rpm for 2 min. Eight microliter of supernatant was electrophoresed in 1% NuSieve 3:1 agarose and visualized after staining with ethidium bromide. Direct PCR was done using one of white colonies with internal primers under the above mentioned Nested-PCR conditions. The insert SREHP gene was also digested from the recombinant pBSc with BamH1 enzyme (McPherson and Moller, 2000; Sambrook *et al*., 2001).

RESULTS

Amplification of HM-1: IMSS with *E. histolytica* specific primers (HSP1, HSP2) gave the expected product of 340-bp. Iranian *E. histolytica/E. dispar* isolate (SHR 10 IR) showed a fragment of about 340 bp and identified as *Entamoeba histolytica* strain.

Both HM-1: IMSS cl6 and SHR 10 IR strains failed to amplify with *Entamoeba dispar* specific primers (DSP1, DSP2), while *E. dispar* AS 16 IR strain (Kobayashi *et al*.,

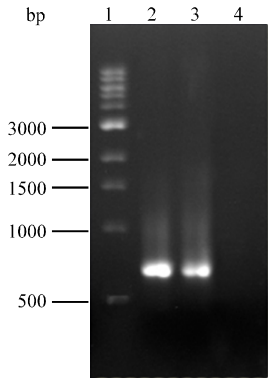

Fig. 1: Agarose gel electrophoresis of SREHP by Nested-PCR from Iranian Entamoeba histolytica isolate
Lane 1: 1 kb DNA ladder
Lane 2: SHR 10 IR isolate
Lane 3: positive control (HM1-IMSS)
Lane 4: negative control (DW)

2005) gave an expected band of about 430 bp as positive control. Nested-PCR product of SREHP gene gave a band of about 700 bp (Fig. 1).

Restriction enzyme analysis of recombinant plasmid by BamH1 showed a band of the same size of the Nested-PCR product (about 700 bp) (Fig. 2). Direct PCR from

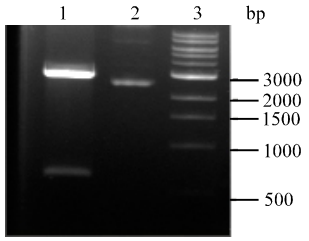

Fig. 2: Agarose gel electrophoresis of digested SREHP gene from recombinant pBSc
Lane1: Enzyme digested recombinant pBSc shows a band of about 700-bp and the linear pBSc
Lane 2: The recombinant pBSc
Lane 3: 1 KbDNA ladder

white colony was also approved the cloned SREHP gene. Finally DNA fragment of cloned SREHP was excised from recombinant plasmid, sequenced and an Open Reading Frame (ORF) of 666 bp was identified (Fig. 3).

DISCUSSION

In the present study Serine- rich *E. histolytica* Protein gene of Iranian *E. histolytic*a isolate was cloned and sequenced. In fact *E. coli* contained recombinant plasmid is the source for expression and preparation of SREHP

ATGTTCGCAT TTTTATTGTT TATTGCATTC ACTAGTGCAA CTAATATCAT TCTTGATTTG
GATCAAGAAG TAAAAGATAC TAATATCTAC GGTGTATTTT TAAAGAACGA AGCTAGTCCT
GAAAAGCTTG AAGAAGCTGA AGAAAAAGAA AAAAGTAGCT CAGCAAAACC AGAATCAAGT
TCAAATGAAG ATAATGAAGA TGATGAAGAT **GAAAAAGCAA GTTCAAGTGA TAACTCA**GAA
TCAAGCTCAA GTGATAAACC **AGATAATAAA CCA**GAAGCAA GTTCAAGTGA TAAACCA**GAA**
GCAAGTTCAA GTGATAAACC AGAAGCAAGT TCAAGTGATA AACCA**GAAGT AAGTTCAAGT**
GATAAACCAG ATAATAAACC A**GAAGCAAGT TCAAGTGATA AACCA**GATAA TAAACCA**GAA**
GCAAGCTCAA GTGATAAACC AGATAATAAA CCA**GAAGCAA GCTCAAGTGA TAAACCA**GAT
AATAAACCA**G AAGCAAGCTC AACTAATAAA CCA**GAAGCAA GCTCAACTAA TAAACCA**GAA**
GCAAGCTCAA CTAGTAATTC AAATGATAAA TCAGGAAGTA GTTCAGATAA CGATAATAAT
AACCTTGATG CTGCATCAAG TCCATTCATT GTTTTCTGTG CTATCATTAT AGCTATCATC
TTCTAA.

Fig. 3: Sequencing result of SREHP gene from Iranian *E. histolytica* isolate (SHR 10 IR). The repeat-containing region of the gene shown with rectangle

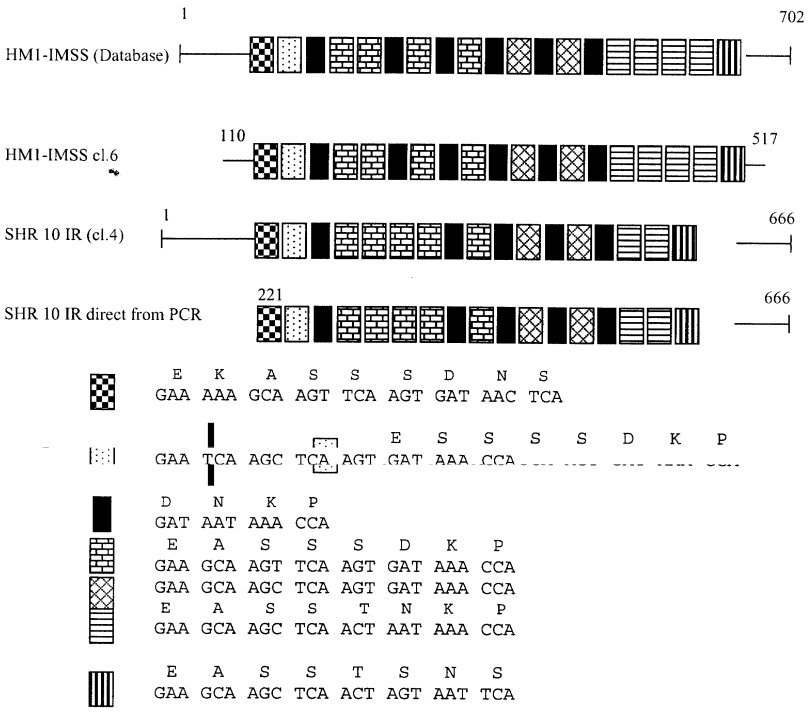

Fig. 4: Schematic representation of polymorphism in the repeat-containing region of the SREHP gene among SHR 10 IR and HM1-IMSS isolates. The numbers shown in the figure correspond to nucleotide numbers of SREHP of HM1-IMSS (GenBank /EMBL/DDBJ accession number M80910). Nucleotide and deduced amino acid sequences of tetra-, octa- and nonapeptide repeats are also shown

recombinant protein that is useful in diagnosis and prevention of amoebiasis.

SREHP gene sequences contain conserved regions and internal tandem dodecapeptides and octapeptides repeat units (Haghighi *et al*., 2002). The 666 base pair SREHP gene of SHR 10 IR isolate was different from standard HM1-IMSS cl.6 strain as well as all previously reported *E. histolytica* genotypes (Fig. 4) (Clark and Diamond, 1993; Haghighi *et al*., 2002, 2003). Antigenic variations in lengths, types and numbers of internal repeat units were observed in SREHP gene of SHR 10 IR isolate. Polymorphisms in lengths, types and numbers of internal repeat units were previously observed in locus 1-2 as well as in the repeat-containing region of serine-rich *E. histolytica* protein gene (Haghighi *et al*., 2002 and 2003). Genotyping of the *E. histolytica* isolates could serve as a tool to fingerprint individual isolates and should help to determine geographic origin of isolates and routes of transmission (Haghighi *et al*., 2002).

A WHO expert consultation on amoebae stressed the need for development for specific diagnosis of *E. histolytica* pathogen and *E. dispar* nonpathogen in clinical and epidemiological studies (WHO/PAHO/ UNESCO, 1997). *E. histolytica* is rare in central of Iran and *E. dispar* is the predominant species (96.9%) (Hooshyar *et al*., 2003). Therefore in most cases anti-parasitic therapy is not necessary. Today antigen based ELISA kits (*E. histolytica* test II; TechLab, Blacksburg, Va.) or monoclonal antibodies against the serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim- Hersel, Germany) are reported to be specific for *E. histolytica* diagnosis (Tanyuksel *et al*., 2003). Preparation of recombinant protein such as Merlin Diagnostika kit for proper diagnosis of *E. histolytica* is necessary, for avoiding inappropriate usage of drug and drug resistance (Tanyuksel *et al*., 2003). The purified recombinant SREHP as the target antigen in an ELISA test could prove useful in serodiagnosis of acute invasive amoebiasis (Myung *et al*., 1992). Considering WHO recommendation on necessity of molecular studies on *E. histolytica* specific immunogenic compound, proteins associated with virulence have been identified, including lectin that mediates adherence to epithelial cells and SREHP are potential targets for anti amoebic vaccines. SREHP is a protective antigen and is a major candidate of vaccine for preventing of amebic liver abscess (Zhang and Cieslak, 1994a). Successful expression of Serine rich *E. histolytica* protein in an attenuated vaccine strain

Salmonella typhi, TY2 chi4297, combination vaccine designed to prevent both amoebiasis and typhoid fever was described (Zhang and Stanely, 1997). Cieslak demonstrated the feasibility of expressing recombinant amoebic proteins in attenuated *S. typhimurium* stains and a successful vaccination of animals with recombinant *E. histolytica* antigen was reported (Cieslak *et al*. 1993; Zhang and Cieslak, 1994b). This is the first report of cloning SREHP gene in Iran. The ability to isolate *E. histolytica* specific gene and to express the genes may provide useful reagents for studying the molecular basis of *E. histolytica* pathogenesis and future development of vaccine.

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