Genetic characterization of Entamoeba histolytica reveals a significant association of parasite genotype with disease outcome

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ABSTRACT
Amoebiasis caused by the enteric parasite \textit{Entamoeba histolytica} has variable disease outcomes. The association between parasite genotypes and outcome of amoebic infection still remains obscure and requires to be explored. Genetic characterization of clinical isolates is certainly a useful method to explore this relation. \textit{E. histolytica} isolates from different disease outcomes has been genetically characterized based on the widely used repetitive marker, Serine rich \textit{E. histolytica} protein gene (\textit{Srehp}). Genetic organizations of our study isolates are unique in compare to previous reports. Most of the identified repeat patterns are outcome specific. Significant association between parasite genotypes and outcome of amoebic infection has also been established.

Indexing terms/Keywords
\textit{Entamoeba histolytica}; Genotyping; Serine rich \textit{E. histolytica} protein gene (\textit{Srehp}); Disease outcome; Phylogeny

Academic Discipline And Sub-Disciplines
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SUBJECT CLASSIFICATION
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TYPE (METHOD/APPROACH)
Medical research

INTRODUCTION
Amoebiasis caused by the gastrointestinal parasite \textit{Entamoeba histolytica} is one of the major parasitic diseases after Malaria and is responsible for approximately 100,000 human deaths per annum (WHO, 1997). The parasite has an interchangeable two stage life cycle consisting of an infective cyst form and a motile pathogenic trophozoite form. Infection is endemic in many developing countries where poor sanitation and malnutrition are common. Infection can also be restricted to certain population in some developed countries (among male homosexual population in Japan, both homo and hetero sexual population in Canada) (Haghighi et al., 2002; 2003; Salit et al., 2009). The outcomes of \textit{E. histolytica} infection are highly variable. Majority of infected individuals remain asymptomatic. Only a fraction of the infected develops diarrhea, dysentery, and rare extra-intestinal complications like- amoebic liver abscess (ALA) (Stanley 2003; Mehmet and Petri 2003). Specific determinant for these diverse disease outcomes still remains elusive; however, host genetics and parasite genotypes could be possible factors. (Ali et al., 2007; Duggal et al., 2004). Proper identification and genetic characterization of infecting strains from endemic areas throughout the world are certainly efficient to explore the hidden genetic traits of parasite directly linked to its virulence or associated with disease outcome. Selection of suitable genetic markers is needed for an optimum genotyping system. The \textit{E. histolytica} genome does not appear to contain microsatellites. Therefore, measurement of genetic diversity and estimation of population structures has relied upon other polymorphic markers, like serine-rich \textit{E. histolytica} Protein gene (\textit{Srehp}) (Ayeh-Kumi et al., 2001; Haghighi et al., 2002, 2003, 2008; Simonishvili et al., 2005; Rivera et al., 2006; Samie et al., 2008) and Chitinase gene (Haghighi et al., 2002; 2003). Serine rich \textit{E. histolytica} protein has antigenic properties, involves in phagocytosis of apoptotic host cells to prevent inflammatory responses by the host (Teixeira et al., 2008). \textit{Srehp} locus contain tandem repeats, which shows high degree
of inter isolate polymorphism based on their repeat type, number and arrangement patterns (Haghighi et al., 2002; Ghosh et al., 2000). In the present study genotyping of E. histolytica clinical isolates, obtained from the individual with different diseases outcomes were performed using Srehp locus as genetic marker. The goals of our study were to determine the genetic pattern and the diversity of E. histolytica clinical isolates based on specific genetic marker and to identify any significant association of parasite genotypes with the disease outcomes. Efforts were also made to determine a possible phylogenetic relation among repeat patterns of target locus, obtained from the genetic analysis of our study isolates. High degree of genetic diversity was observed among E. histolytica isolates and repeat patterns exhibiting significant association with a particular disease outcome were also identified.

2. MATERIAL AND METHODS

2.1 Sample Collection and Detection of Entamoeba histolytica.

A total of 51 Indian E. histolytica isolates from different disease outcomes were included in the present study. Among them, 26 were from diarrheal cases, 20 from Liver abscess cases and 5 from asymptomatic cases. The diarrheal fecal samples were obtained from the patients admitted in Infectious Diseases Hospital, Kolkata with sole diarrheal complications. These patients were mostly from “low socioeconomic communities of Kolkata” where poor hygiene, sanitation and malnutrition were common (Mukherjee et al., 2009). Asymptomatic samples were also collected from the same “low socioeconomic communities” through an on-going field project studying the parasite burden of those communities. The Liver aspirate samples were obtained from Amoebic Liver Abscess (ALA) patients admitted to various hospitals in Kolkata. The individuals with different amoebic symptoms were screened based on the predetermined inclusion and exclusion criteria. The study received an ethical clearance from the NICED IEC (i.e. National Institute of Cholera and Enteric Diseases Institutional Ethical Committee). Informed consent was obtained from the patients (in case of children, consent was obtained from their parents). The parasites were primarily detected by conventional microscopy and Antigen capture ELISA (E HISTOLYTICA II, TECHLAB, USA) followed by genetic identification through PCR amplification of E.histolytica SSU rRNA gene (Mukherjee et al., 2009). DNA was isolated directly from clinical samples using STOOL DNA Minikit (QIAGEN, USA) as per manufacturer’s protocol.

2.2. PCR amplification, DNA sequencing and analysis.

Nested PCR amplification was performed with E.histolytica specific primer pairs, targeting a part of Srehp locus (Ghosh et al., 2000; Haghighi et al., 2002) (Table 1). The reaction was carried out in 50 µl reaction volume containing approximately 0.4 µg and 0.1 µg of template DNA for primary and nested PCR respectively, 10 µM of each primer, 1.5 mM MgCl₂, 1 µg of Bovine Serum Albumin (SIGMA, USA), 200 µM dNTP and 2.5 U of Taq DNA polymerase (Bioline, USA) with the reaction parameters as initial denaturation for 15 mins or 4 mins (Primary and Nested respectively) at 95°C. This was followed by 30 cycles of denaturation at 94°C for 30 secs, annealing at 57°C for 30 secs, extension at 72°C for 1 min. This was followed by the final extension for 10 mins at 72°C.

The amplified PCR products were separated, according to their size by electrophoresis on 1.5% agarose gels (SIGMA, USA). Amplicons of the expected sizes were extracted from gels and purified (ROCHE, Germany), and their yield was verified again by electrophoresis. Purified PCR products were sequenced directly with specific primers (marked with * in table 1) using the ‘BigDye Terminator’ V3.1 cycle sequencing kit’ (APPLIED BIOSYSTEMS, USA) as per the manufacturer’s protocol. The labeled DNA fragments were purified by sodium acetate and ethanol precipitation. The sequencing was performed in an ABI 310 PRISM Automated Genetic Analyzer. The nucleotide sequences were then analyzed with the tandem repeat finder software (http://tandem.bu.edu/trf/trf.html) (Benson, 1999) and each unique repeat unit was coded with a particular color for better understanding (Figure 1). The identified repeat patterns that had been reported previously (Haghighi et al., 2002; Ghosh et al., 2000) were named according to their existing nomenclature. Newly identified repeat patterns were assigned by alphanumerical codes beginning with ‘IND’ (to indicate their Indian origin) followed by ‘repeat pattern number’ (i.e. 1, 2, 3,… etc.) and ‘name of corresponding locus’ (i.e. Srehp). All the sequences obtained from the study were submitted to NCBI GenBank with accession numbers KF355999- KF356022, JN984716- JN984744. The sequences of all representative repeat patterns from target locus were aligned using ClustalW multiple alignment program of MEGA Version 4 software (Tamura et al., 2007) and edited manually. The alignment thus obtained was used for constructing phylogenetic tree through “Generalized Time Reversal (GTR) + gamma” substitution model of SeaView Graphical Interface Version 4 software (Gouy et al., 2010). Association of repeat patterns with specific clinical outcome was evaluated by Epi-Info ver 3.5.4 software (Dean et al., 2007).

3. RESULT

3.1 Repeat polymorphism in Serine rich E. histolytica protein gene (Srehp).

Sequence analysis of amplified PCR products revealed a high degree of polymorphism within the Srehp locus among our 51 study isolates. A total of 24 different repeat patterns were observed. All of them were newly identified, unique to Indian isolates and assigned as Indian Srehp 1 to 24 (i.e. IND1SRP to IND24SRP) (Figure 1). Repeat pattern information of target locus (i.e. Srehp) of all (51) study isolates has been provided in table 2.

3.2 Distribution of Repeat patterns among three different disease outcomes.

Majority of identified repeat patterns of Srehp locus were exclusive for a particular disease outcome. For example IND12SRP - IND15SRP, IND17SRP - IND24SRP were found only in liver abscess (LA) outcome, whereas IND2SRP and
IND4SRP – IND10SRP were only present in diarrheal (D) outcome (Table 3). In contrast, only few repeat patterns were common among different disease outcomes. For example, IND1SRP, IND11SRP were found in both LA and D outcomes. Similarly, IND16SRP was present in both LA and asymptomatic (AS) outcomes and only one repeat pattern (i.e. IND3SRP) was present in all three disease outcomes (i.e. D, LA and AS) (Table 3).

3.3. Association between repeat patterns and outcome of infection.

One repeat pattern of Srehp locus (i.e. IND3SRP) showed significant associations with disease outcomes. It showed a significant positive association with diarrhea (Co-efficient value = 0.481, p value = 0.0003), but a significant negative association with liver abscess (Co-efficient value = -0.313, p value = 0.0292) (Table 4).

3.4. Phylogenetic analysis.

Phylogenetic analysis has identified a separate cluster of repeat patterns, exclusive for liver abscess outcome. The cluster was marked with green colour (Figure 2).

Table 1: List of gene specific primer, used in the study.

| Gene name                      | PCR round | Primer name | Primer sequence (5’to 3’)                  |
|--------------------------------|-----------|-------------|--------------------------------------------|
| **Serine rich E. histolytica protein gene (Srehp)** | Primary   | SREHP F1    | GCTAGTCCTGAAAAAGCTTGAGAAAGCTG               |
|                                |           | SREHP R1    | GGAATCTGATCGACGATCAAGGT                    |
|                                | Nested    | SREHP F2*   | TATTATTTATTATCGTATCTGACTACTTCCTG           |
|                                |           | SREHP R2*   | TGAAGATAATGAAAGATGAGATGAGAGT               |

* Gene specific primer pairs used for sequencing of amplified PCR products.

Figure 1: Schematic representation of repeat patterns of Serine rich E. histolytica protein gene (Srehp) based on the nucleotide sequences of our study isolates: repeat units are depicted in rectangles (with specific colour coding) and non-repeat regions are shown in lines. Newly identified repeat patterns were assigned by alphanumerical codes beginning with ‘IND’ (to indicate their Indian origin) followed by ‘repeat pattern number’ (i.e. 1, 2, 3,… etc.) and ‘name of corresponding locus’ (i.e. Srehp or SRP).
Table 2: Detailed repeat pattern information of *Srehp* locus of our study isolates.

| Sl. No. | Isolate | Clinical symptoms | Serine rich *E. histolytica* protein gene (*Srehp*) |
|---------|---------|-------------------|---------------------------------------------|
| 1       | LA14    | Liver abscess     | IND1SRP                                   |
| 2       | LA17    | Liver abscess     | IND11SRP                                  |
| 3       | LA25    | Liver abscess     | IND12SRP                                  |
| 4       | LA35    | Liver abscess     | IND17SRP                                  |
| 5       | LA18    | Liver abscess     | IND3SRP                                   |
| 6       | LA10    | Liver abscess     | IND24SRP                                  |
| 7       | LA11    | Liver abscess     | IND16SRP                                  |
| 8       | LA7     | Liver abscess     | IND18SRP                                  |
| 9       | LA9     | Liver abscess     | IND13SRP                                  |
| 10      | LA6     | Liver abscess     | IND19SRP                                  |
| 11      | LA5     | Liver abscess     | IND14SRP                                  |
| 12      | LA3     | Liver abscess     | IND22SRP                                  |
| 13      | LA2     | Liver abscess     | IND20SRP                                  |
| 14      | LA1     | Liver abscess     | IND21SRP                                  |
| 15      | LA29    | Liver abscess     | IND3SRP                                   |
| 16      | LA28    | Liver abscess     | IND11SRP                                  |
| 17      | LA34    | Liver abscess     | IND16SRP                                  |
| 18      | LA27    | Liver abscess     | IND17SRP                                  |
| 19      | LA33    | Liver abscess     | IND23SRP                                  |
| 20      | LA16    | Liver abscess     | IND15SRP                                  |
| 21      | G12     | Asymptomatic      | IND3SRP                                   |
| 22      | G48     | Asymptomatic      | IND3SRP                                   |
| 23      | G224    | Asymptomatic      | IND3SRP                                   |
| 24      | G70     | Asymptomatic      | IND3SRP                                   |
| 25      | G83     | Asymptomatic      | IND16SRP                                  |
| 26      | ID1290  | Diarrhea          | IND1SRP                                   |
| 27      | ID1098  | Diarrhea          | IND2SRP                                   |
| 28      | ID1450  | Diarrhea          | IND3SRP                                   |
| ID   | Pattern | Disease | Repeat Pattern |
|------|---------|---------|----------------|
| 29   | ID1393  | Diarrhea| IND3SRP        |
| 30   | ID1301  | Diarrhea| IND4SRP        |
| 31   | ID1695  | Diarrhea| IND5SRP        |
| 32   | ID1093  | Diarrhea| IND3SRP        |
| 33   | ID568   | Diarrhea| IND3SRP        |
| 34   | ID1234  | Diarrhea| IND8SRP        |
| 35   | ID2618  | Diarrhea| IND3SRP        |
| 36   | ID1873  | Diarrhea| IND3SRP        |
| 37   | ID1606  | Diarrhea| IND3SRP        |
| 38   | ID796   | Diarrhea| IND9SRP        |
| 39   | ID754   | Diarrhea| IND3SRP        |
| 40   | ID727   | Diarrhea| IND10SRP       |
| 41   | ID916   | Diarrhea| IND3SRP        |
| 42   | ID1027  | Diarrhea| IND3SRP        |
| 43   | ID1328  | Diarrhea| IND11SRP       |
| 44   | ID1721  | Diarrhea| IND3SRP        |
| 45   | ID1980  | Diarrhea| IND1SRP        |
| 46   | ID1678  | Diarrhea| IND7SRP        |
| 47   | ID2014  | Diarrhea| IND3SRP        |
| 48   | ID2172  | Diarrhea| IND3SRP        |
| 49   | ID1462  | Diarrhea| IND1SRP        |
| 50   | ID2134  | Diarrhea| IND1SRP        |
| 51   | ID2234  | Diarrhea| IND6SRP        |

Table 3: Distribution of repeat patterns among disease outcomes.

| Serine rich *E. histolytica* protein gene (*Srehp*) |
|----------------------------------------------------|
| Common patterns *a*                                  |
| IND3SRP                                            |
| Sole D patterns *b*                                 |
| IND2SRP                                            |
| IND4SRP                                            |
| IND5SRP                                            |
| IND6SRP                                            |
| Locus | Repeat pattern | Liver abscess (LA) | Diarrheal (D) | Asymptomatic (AS) |
|-------|----------------|-------------------|--------------|------------------|
| Serine rich *E. histolytica* protein gene (*Srehp*) | IND3SRP | Co-eff*: -0.313, p\textsuperscript{b} = 0.0292 | Co-eff*: 0.481, p\textsuperscript{b} = 0.0003 | X\textsuperscript{c} |

\textsuperscript{a} Repeat patterns found in all three outcome groups (i.e.- Diarrheal, Liver abscess and Asymptomatic), \textsuperscript{b} Probability value of the particular association, \textsuperscript{c} Does not have any significant association with disease outcomes.

Table 4: Repeat patterns of target locus, showing significant association with disease outcomes.
4. DISCUSSION

Information regarding the genetic organization of infecting strains from the endemic areas throughout the world is needed to understand the role of parasite genome in the outcome of amoebic infection (Weedall and Hall, 2011). Since, India has been considered as a country with highest prevalence of *E. histolytica* infection (Hughes and Petri, 2000), genetic characterization of *E. histolytica* clinical isolates from India is also a certainly necessary assignment. Since, the genome of this fascinating parasite does not appear to contain microsatellites, genetic characterization of clinical isolates has been usually performed based on other genetic markers like serine-rich *E. histolytica* Protein gene (*Srehp*) (Ayeh-Kumi et al., 2001; Haghighi et al., 2002, 2003, 2008; Simonishvili et al., 2005; Rivera et al., 2006; Samie et al., 2008) and Chitinase gene (Haghighi et al., 2002; 2003). *Srehp* locus exhibits comparatively high degree of inter-isolate polymorphism than Chitinase (Haghighi et al., 2002).

Sequence analysis of our study isolates has also identified a high degree of inter-isolate polymorphism in *Srehp* locus. As much as 24 different repeat patterns were observed. Moreover, all of them were newly identified and unique to Indian isolates. Since SREHP is an immunogenic surface antigen, such high degree of genetic diversity could be due to the host selection pressure on parasite during immune evasion (Das and Ganguly, 2014). Distribution of repeat patterns among disease groups has also revealed an interesting scenario. Most of identified repeat patterns were outcome specific. Only a single repeat pattern (i.e. IND3SRP) was found in all three disease outcomes (i.e. in D, LA and AS). Such outcome
specific distribution of repeat patterns could highlight the possibility of their associations with the disease outcomes. The association between the repeat pattern and disease outcome were further analyzed to justify this observation. It revealed that repeat pattern which showed a significant positive association with D (diarrhea), showed a significant negative association with LA (liver abscess) (in case of IND3SRP). Hence, isolates from diarrheal and liver abscess outcomes may have two distinct genetic organizations. However, phylogenetic analysis has a moderate resolution, showed a distinct cluster of repeat patterns exclusive for liver abscess outcome.

5. CONCLUSION

The present study has unveiled the genetic organization of Indian E. histolytica isolates from different disease outcomes. The results of this study also support the hypothesis that a relationship exists between the parasite genotypes and outcomes of amoebic infection. However, detailed multilocus sequence typing of E. histolytica isolates from different disease outcomes should be carried out to identify the exact genetic traits of parasite affecting its virulence capacity as well as different disease causing abilities.

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Prof. Tomoyoshi Nozaki has been working as a Director of National Institute of Infectious Diseases, Japan. His major area of interest is pathogenesis of parasitic protozoa Biochemistry of sulfur-containing metabolism of parasitic protozoa and drug development Vesicular trafficking of virulence factors and phagocytosis and also he is working on evolution and function of mitochondrion-related organelles in anaerobic protozoa.

Dr. Sandipan Ganguly has been working as a Head of the Division of Parasitology in the National Institute of Cholera and Enteric Diseases, Kolkata. He has been working on various aspects of enteric parasitology with special emphasis on molecular and cellular aspects of pathogenesis in enteric parasites. Study the effects of oxidative stress on microaerophilic Giardia at its cellular, genome, proteome and metabolomic level, molecular diagnosis of enteric parasites, molecular epidemiology of Coccisides and other enteric parasites in immunocompromized patients.protozoa.