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

Authors:

Koushik Das[a], Punam Chowdhury[a], Tomoyoshi Nozaki[b] and Sandipan Ganguly[a]*

Affiliation:

a) Division of Parasitology, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, Kolkata 700010, India

b) Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

E-mail address:

kaushikdas55@gmail.com

punam.bt07@gmail.com

nozaki.niid@go.jp

S Ganguly: sandipanganguly@hotmail.com (*corresponding author)

ABSTRACT

Amoebiasis caused by the enteric parasite *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. *E. histolytica* isolates from different disease outcomes has been genetically characterized based on the widely used repetitive marker, Serine rich *E. histolytica* protein gene (*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

Entamoeba histolytica; Genotyping; Serine rich *E. histolytica* protein gene (*Srehp*); Disease outcome; Phylogeny

Academic Discipline And Sub-Disciplines

Life Science and Parasitology

SUBJECT CLASSIFICATION

Molecular Epidemiology

TYPE (METHOD/APPROACH)

Medical research

INTRODUCTION

Amoebiasis caused by the gastrointestinal parasite *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 *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 *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 *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). Serine rich *E. histolytica* protein has antigenic properties, involves in phagocytosis of apoptotic host cells to prevent inflammatory responses by the host (Teixeira *et.al*, 2008). *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 pM of each primer, 1.5 mM $MgCl_2$, 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 [a] 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	GCTAGTCCTGAAAAGCTTGAAGAAGCTG
		SREHP R1	GGACTTGATGCAGCATCAAGGT
	Nested	SREHP F2[a]	TATTATTATTATCGTTATCTGAACTACTTCCTG
		SREHP R2[a]	TGAAGATAATGAAGATGATGAAGATG

[a] Gene specific primer pairs used for sequencing of amplified PCR products.

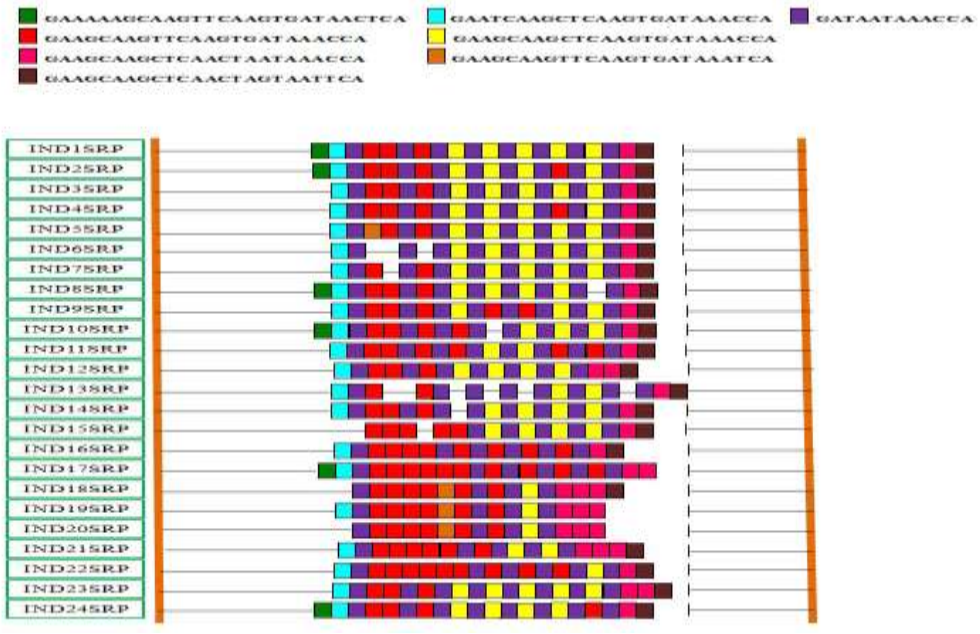

Figure 1: Schematic representation of repeat patterns of Serine rich *E. histolytica* protein gene (*Srehp*) based on the nucleotide sequences of our study isolates: repeat units are depicted in rectangles (with specific colour coding) and non-repeat regions are shown in lines. Newly identified repeat 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		Serine rich *E. histolytica* protein gene (*Srehp*)
	Name	Clinical symptoms	
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

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

	IND7SRP
	IND8SRP
	IND9SRP
	IND10SRP
Sole LA patterns [c]	**IND12SRP**
	IND13SRP
	IND14SRP
	IND15SRP
	IND17SRP
	IND18SRP
	IND19SRP
	IND20SRP
	IND21SRP
	IND22SRP
	IND23SRP
	IND24SRP
Sole AS patterns [d]	**X[i]**
D+LA patterns [e]	**IND1SRP**
	IND11SRP
LA+AS patterns [f]	**IND16SRP**
D+AS patterns [g]	**X[i]**

[a] Repeat patterns found in all three outcome groups (i.e.- Diarrheal, Liver abscess and Asymptomatic), [b] Repeat patterns found only in Diarrheal outcome, [c] Repeat patterns found only in Liver abscess outcome, [d] Repeat patterns found only in Asymptomatic outcome, [e] Repeat patterns found in both Diarrheal and Liver abscess outcomes, [f] Repeat patterns found in both Liver abscess and Asymptomatic outcomes, [g] Repeat pattern found in both Diarrheal and Asymptomatic outcomes, [i] No repeat patterns found in particular outcome groups.

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

Locus	Repeat pattern	Liver abscess (LA)	Diarrheal (D)	Asymptomatic (AS)
Serine rich *E. histolytica* protein gene (*Srehp*)	IND3SRP	Co-eff[a]: -0.313 p[b] = 0.0292	Co-eff[a]: 0.481 p[b] = 0.0003	X[c]

[a] Correlation co-efficient value of the particular association indicate whether the association is positive or negative, [b] Probability value of the particular association, [c] Does not have any significant association with disease outcomes.

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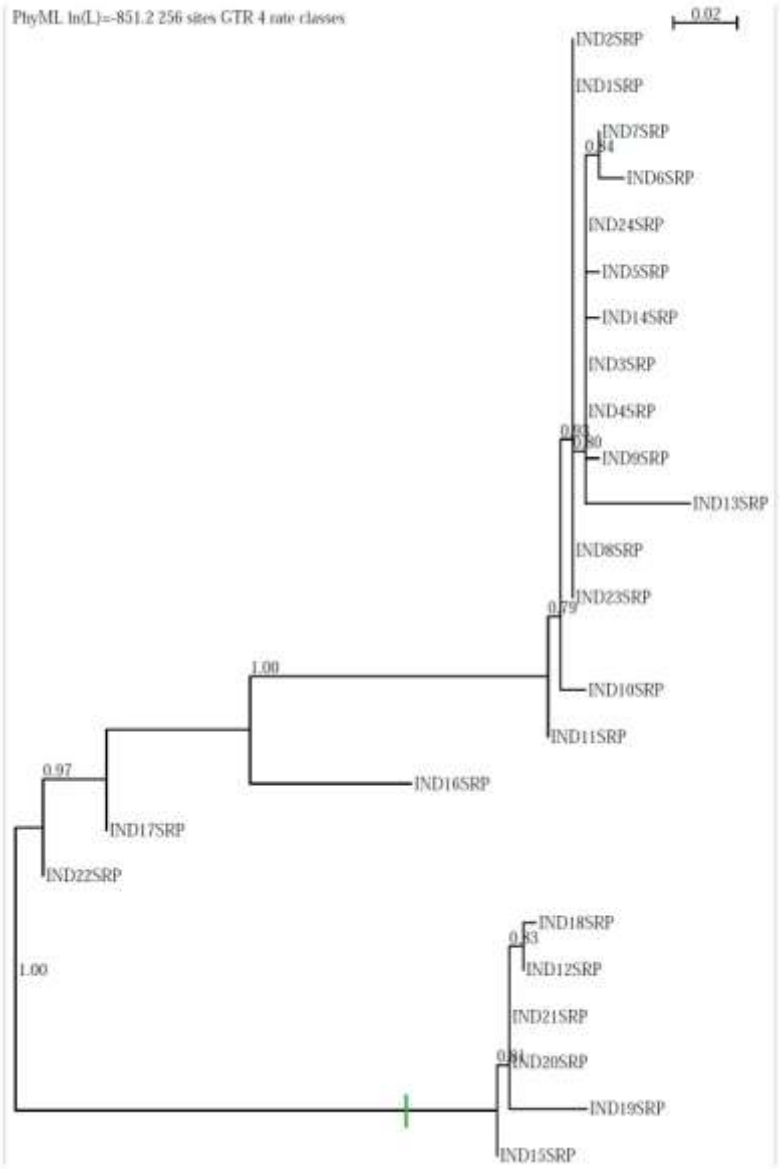

Figure 2: Phylogenetic relation among repeat patterns of Serine rich *E. histolytica* protein gene (*Srehp*): The sequences of repeat patterns from *Srehp* gene were aligned using ClustalW multiple alignment program of MEGA Version 4 software. Phylogenetic tree was constructed from the alignment through SeaView Graphical Interface Ver.4 software using a maximum likelihood matrix algorithm. A separate cluster of repeat patterns, exclusive for liver abscess (LA) outcome has been identified. The cluster was marked with green colour.

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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Author' biography with Photo

Koushik Das has been working in the National Institute of Cholera and Enteric Diseases, Kolkata as a Senior Research Fellow under the supervision of Dr. Sandipan Ganguly. He is interested on the genetic variability of *Entamoeba histolytica* to find out the association of parasite genotype with disease outcome.

Punam Chowdhury has been working in the National Institute of Cholera and Enteric Diseases, Kolkata as a Senior Laboratory Technician under the supervision of Dr. Sandipan Ganguly. She is interested on the parasite detection and genotyping to find out the association of parasite genotype with disease outcome.

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 pathogenesity 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.