Identification and Molecular Characterization of Common Enteric Parasites in Kolkata with Special Reference to *Entamoeba* spp

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PREFACE

This Ph.D. thesis was prepared at the **Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, India** to fulfil the requirements for obtaining a Ph.D. degree. The thesis is titled "**Identification and Molecular Characterization of Common Enteric Parasites in Kolkata with Special Reference to *Entamoeba* spp**" and the research was conducted at **ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India**. The thesis was completed under the direct guidance of **Dr. Sandipan Ganguly**, Ph.D., Senior Deputy Director (Scientist F) and Head, Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India.

This thesis has been solely composed by the candidate and has not been submitted for any other degree, except where specifically acknowledged.

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Dedicated to all those who relentlessly chasing their dreams and to those who unwaveringly stand by their side, aiding them in their pursuit of success....

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ABBREVIATIONS

Abbreviation	Meaning
Mb	Megabase
mg	Milligram
min	Minute
ml	Milliliter
mmol	Millimol
mRNA	Messenger RNA
ng	Nanogram
nm	Nanometer
PBS	Phosphate Buffered Saline
rDNA	Ribosomal DNA
RFLP	Random Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolution per Minute
rRNA	Ribosomal RNA
RT	Reverse Transcription
sec	Second
SSU	Small Subunit
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TE	Tris EDTA
UV	Ultraviolet
IDBG	Infectious Diseases & Beliaghata General
tRNA	Transfer RNA
STR	Short Tandem Repeat
CHI	Chitinase
SNP	Single Nucleotide Polymorphism
SREHP	Serine Rich *E. histolytica* Protein
AS	Asymptomatic
D	Diarrhoea
LA	Liver Abscess
ICMR-NICED	ICMR-National Institute of Cholera and Enteric Diseases
IEC	Institutional Ethical Committee
GTR	Generalized Time Reversal
kerpl	Lysine and Glutamic Acid-Rich Protein Gene 1
kerp2	Lysine and Glutamic Acid-Rich Protein Gene 2
apc	Amoebapore C
POR	Polymerase Chain Reaction
LD	Linkage Disequilibrium
MLGs	Multi-Locus Genotypes

Abbreviation	Meaning
S	Number of Polymorphic/Segregating Sites
h	Number of Haplotypes
K	Average Number of Nucleotide Differences
Hd	Haplotype Diversity
π	Nucleotide Diversity
Sole D	Diarrheal Patients Solely Infected with *E. moshkovskii*
IEH	*E. moshkovskii* Positive Samples Co-Infected with *Entamoeba histolytica*
IOEP	*E. moshkovskii* Positive Samples Co-Infected with Other Enteric Parasites (e.g., *G. lamblia, Cryptosporidium* spp)
ISTH	*E. moshkovskii* Positive Samples Co-Infected with Soil-Transmitted Helminths
IB/V	*E. moshkovskii* Positive Samples Co-Infected with Other Diarrhea-Causing Bacteria (e.g., *E. coli, Shigella spp, V. cholera*) or Virus (Rotavirus)
P	Correlation Coefficient Value of the Particular Association
df	Degree of Freedom
X^2	Chi-Square Value
α	Alpha
β	Beta
µm	Micrometer
µl	Microliter
bp	Base pair
dNTP	Centimetre
ELISA	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
Kb	Kilobase

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ABSTRACT

Identification and Molecular Characterization of Common Enteric Parasites in Kolkata with Special Reference to *Entamoeba* spp

Diarrheal diseases are a major health concern, affecting millions of people worldwide every year and causing a high rate of illness and death. The three most common parasites that cause diarrhoea are *Entamoeba* spp, *Giardia* spp, and *Cryptosporidium* spp. In this study, we have identified and characterized the most prevalent enteric parasites responsible for diarrhoea in Kolkata and adjacent areas, with a specific focus on amoebic infection. The primary causative agent for amoebic infection is *Entamoeba histolytica*. Amoebic infection is a complex issue as there are several species that are morphologically indistinguishable from each other, including *E. dispar, E. bangladeshi*, and *E. moshkovskii*. Cysts of the other non-pathogenic amoeba *E. hartmanni* also can be misidentified as the pathogenic *E. histolytica* under a microscope. This makes it challenging to accurately estimate the prevalence of each species and its potential to cause disease in humans.

Over a period of three years, we conducted active surveillance among 6051 patients with diarrhoea in Kolkata, India, to study the prevalence, genetic and phylogenetic structure of the common enteric parasites population. We employed microscopy, PCR, DNA sequencing, and various tools such as MEGA X, DnaSP, PopArt, Clustal W, MultAlin, Seaview, and Tandem Repeats Finder for analyzing the sequencing data. We used GraphPad Prism for statistical analysis.

The prevalence study found *Giardia* in 6.18% and *Cryptosporidium* in 4.36% of cases, indicating a high occurrence of both infections. Furthermore, our study revealed that both cryptosporidiosis and giardiasis were significantly more prevalent among children below 4 years of age. During the study, we also conducted genotyping of *Cryptosporidium* spp using the HSP70 gene. Our results reported the presence of *Cryptosporidium viatorum* in eastern India. This emerging enteropathogen was first described in 2012 and has a considerable prevalence rate. Our DNA sequence analysis revealed that *Cryptosporidium hominis* is the most dominant species in the study regions. This study also reported 4.84% of the examined samples were positive for *Entamoeba* spp. Among these amoebic infections, *E. histolytica* accounted for 0.86% of the cases, *E. moshkovskii* for 3.12%, and *E. dispar* for 0.13%, while the remaining 0.73% could not be identified to a specific species. Statistical analysis confirms a significant association ($P<0.0001$) between *E. moshkovskii* and diarrhoeal incidence. These findings underscore the significant prevalence of *E. moshkovskii* as a major parasitic infection in eastern India. Further analysis found that *E. moshkovskii* infection was significantly associated with the age group of 5-12 years. Surprisingly, this parasitic infection exhibits a distinctive seasonal pattern, not previously seen in other parasitic infections. DNA sequencing revealed that the local *E. moshkovskii* strains were 99.59%-100% identical to the prototype (GenBank: KP722605.1). The study found certain SNPs that showed a correlation with clinical features, but it is not necessarily indicative of direct control over pathogenicity. Neutrality tests of different coinfected subgroups indicated deviations from neutrality and implied population expansion after a bottleneck event or a selective sweep and/or purifying selection in co-infected subgroups. The majority of F_{ST} values of different coinfected subgroups were <0.25, indicating low to moderate genetic differentiation within the subgroups of this geographical area. Additionally, we utilized MLST (Multi-Locus Sequence Typing) to identify genetic markers and potential SNPs associated with clinical features in isolates of *E. moshkovskii*. The markers used in our MLST analysis included KERP1, a protein rich in lysine and glutamic acid, as well as amoebapore C (apc) and Chitinases. By integrating these markers into our genotyping system, we were able to identify one genotype (M1) that was significantly associated ($p=0.0394$) with the sole incidence of diarrhoea. We also identified a few potential SNPs in the three genes that were linked to clinical features. Furthermore, using PCR, RFLP, DNA sequencing, and phylogenetic analysis, the true natural animal reservoir of *E. moshkovskii* has been effectively revealed. The findings indicate that pigs serve as the true animal reservoir for this pathogen, with a prevalence rate of 5.4%. This evidence supports the potential for zoonotic transmission of *E. moshkovskii* from pigs to humans. The implications of my study strongly suggest the possibility of such zoonotic transmission, emphasizing the need for further investigation and increased awareness of this potential health risk.

We also employ a strain typing approach in *E. histolytica* isolates that utilize multiple loci, including SREHP and three polymorphic non-coding loci (tRNA linked array N-K2, loci 1-2, and 5-6), for high-resolution analysis. Distinct clinical phenotype isolates underwent amplification and sequencing of studied loci. The nucleotide sequences were analysed using Tandem Repeats Finder to detect short tandem repeats (STRs). This study found significant polymorphism in the size and number of PCR fragments at SREHP and 5-6 locus, while the 1-2 locus and NK2 locus showed variations in PCR product sizes. Out of 41 genotypes, two (I6 and I41) were significantly associated with their respective disease outcomes and were found in multiple isolates. We observed that I6 was linked with a symptomatic outcome, with a statistically significant p-value of 0.0183. Additionally, we found that I41 was associated with ALA disease outcome, with a p-value of 0.0089. Our study revealed new repeat units not previously reported, unveiling the genetic composition of *E. histolytica* strains in India, associated with distinct disease manifestations. Finally, this study has developed a new multiplex PCR and a PCR-RFLP approach, in addition to qPCR, which enables us to accurately detect and distinguish between different parasite species that appear identical. Selecting the appropriate method for identifying and studying parasites is crucial and depends on the intended purpose, available resources, and facilities.

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CHAPTER I

INTRODUCTION

1.1. Introduction

Diarrhoeal diseases affect millions of people worldwide each year and are a leading cause of illness and death. These diseases are prevalent in both developed and developing countries. The primary causes of diarrhoea are viruses, including Norwalk-like viruses, rotaviruses, and enteric adenoviruses, bacteria such as *Campylobacter jejuni*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli*, and cytotoxigenic *Clostridium difficile*, and parasites such as *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*, which are considered to be the most significant causes (Verweij et al. 2004).

According to the World Health Organization (WHO), every year around 50 million people are affected by invasive amoebic infections, leading to 40-100 thousand deaths. These infections are caused by *E. histolytica* cysts, which contaminate food and water. The majority of infected individuals (90%) are asymptomatic carriers, and the parasite resides in their colon, being excreted in stool to complete its life cycle. While most infections do not show any symptoms, 10% of cases result in invasive disease, and in rare instances (<1%, mostly adult males), *E. histolytica* can cause extra-intestinal diseases such as liver abscesses (Ekabe et al. 2021). The mechanism through which *E. histolytica* causes disease involves breaching the intestinal mucosa, invading the underlying lamina propria, and interacting with host immune cells. This triggers a proinflammatory cytokine response, leading to tissue damage and the formation of characteristic amoebic 'flask-shaped ulcers.' It remains unclear why a large proportion of people colonized by *E. histolytica* do not develop the invasive disease (Begum et al. 2021). Host defence mechanisms and pathogen virulence are thought to be the primary controlling factors determining this differential outcome of infection. While some studies have suggested that host immune responses, such as anti-inflammatory cytokines and interleukins, play a critical role in preventing or interrupting tissue invasion, and specific human leukocyte antigen/HLA class-II alleles have been associated with susceptibility to *E. histolytica* infection (Cadiz et al. 2010), the factors that determine the outcome of amoebic infection in the host-parasite relationship remain uncertain. To uncover the hidden genetic traits of the parasite directly linked to virulence or associated with the outcome of amoebic infection, it is essential to identify and genetically characterize infecting strains from endemic areas worldwide.

Amoebic infection is a complex issue as there are several species that are morphologically indistinguishable from each other, including *E. dispar, E. bangladeshi*, and *E. moshkovskii* (Fotedar et al. 2007). This makes it challenging to accurately estimate the prevalence of each

species and its potential to cause disease in humans. Adding to the confusion is the fact that cysts of a non-pathogenic amoeba, *E. hartmanni*, can also be mistaken for the pathogenic *E. histolytica* under a microscope (Burrows et al. 1959). While *E. histolytica* is known to cause fatalities in amoebic infections, the actual prevalence of this species is likely overestimated due to these morphological overlaps. However, recent studies have shown that *E. moshkovskii* may play a more significant role in human infections than previously thought, as it has been detected in multiple countries, including the United States, Italy, Iran, Turkey, Bangladesh, India, Kenya, Australia, Indonesia, Colombia, Malaysia, Tunisia, Tanzania, and Brazil (Ali et al. 2003; Beck et al. 2008; Ngui et al. 2012). Nonetheless, the potential pathogenicity of *E. moshkovskii* in humans remains unclear and requires further investigation.

Cryptosporidium spp and *Giardia lamblia* are two other common enteric protozoan parasites that are responsible for causing gastroenteritis worldwide. These emerging opportunistic pathogens are known to cause diarrhoeal morbidity and are considered a major health concern. There are over 34 known species and 40 genotypes of *Cryptosporidium* that can infect humans and animals, many of which can be transmitted from animals to humans. The most common species causing cryptosporidiosis in humans are *Cryptosporidium hominis* and *Cryptosporidium parvum*, although *Cryptosporidium meleagridis*, *Cryptosporidium felis*, and *Cryptosporidium canis* are also significant causes. Cryptosporidiosis was responsible for about 44.8 million episodes of diarrhoea and 48,300 annual deaths in children under five by 2016, making it the fifth leading cause of diarrhoeal mortality (Kurenzvi et al. 2020). Both *C. parvum* and *Giardia intestinalis* primarily infect children aged 36 months or younger, as these age groups are more susceptible to these diseases. While cryptosporidiosis is typically self-limiting in immunocompetent hosts, it can lead to persistent diarrhoea in children in developing countries and is a major cause of chronic diarrhoea in HIV-positive patients. Giardiasis is also a well-known cause of diarrhoeal illness worldwide and is more commonly found in developing countries. Giardiasis is responsible for over 200 million cases each year globally. Since 2004, the World Health Organization (WHO) has classified *Giardia* as a neglected disease. The prevalence of asymptomatic *Giardia* infection in children ranges from 8% to 30% in developing countries and 1% to 8% in industrialized regions. Individuals with diarrhoea are likely to have a higher incidence of Giardiasis. It spreads via the faecal-oral route from human and animal reservoirs and can cause symptomatic or asymptomatic infections.

The prevalence of enteric parasitic infections that result in diarrhoea is a significant health concern in countries like India that are endemic to these infections. The distribution and

prevalence of these parasites vary in different regions of India. Proper identification and genetic characterization of infecting strains from endemic areas are recommended to explore the genetic traits of parasites that contribute to their virulence or influence the outcome of amoebic infections. Furthermore, there is a lack of research on the population structure and genetic diversity of prevalent enteric parasites in India. The topic of emerging parasites exclusively found in the human gastrointestinal tract that result in severe gastrointestinal illness is an area that has not been thoroughly studied. To address this issue, the present study was conducted with the following objectives:

- Study the prevalence of enteric protozoa in human stool samples in Kolkata and adjacent areas by conventional microscopy and PCR.
- To create a preliminary database on the incidence of enteric protozoan infection in Kolkata and adjacent area
- Determine the genetic pattern and diversity of different local isolates based on specific genetic markers.
- Identification of any significant association of parasites genotype with the disease outcome.
- To determine a possible phylogenetic relation among the study isolates from genetic analysis.
- Molecular Analysis of virulence factors (if any) of the isolates found.
- To understand if amphizoic amoeba *Entamoeba moshkovskii* associated with human pathogenic infections. If it is, to determine the genetic pattern and diversity of clinical isolates of *Entamoeba moshkovskii.*

From March 2017 to February 2020, we conducted a surveillance study in two hospitals in Kolkata, India, namely Infectious Disease & Beliaghata General Hospital and Dr B C Roy Post Graduate Institute of Paediatric Sciences Hospital. This study focused on patients who were admitted to these hospitals with complaints of diarrhoea. These hospitals are well-known referral centres for the treatment of diarrhoeal diseases, in addition to government healthcare facilities. For the purpose of this study, we only included cases of diarrhoea and excluded non-diarrhoeal cases. We identified common enteric parasites such as *E. histolytica*,

Cryptosporidium spp, and *G. lamblia* using the PCR/nested PCR method, which was described by Mukherjee et al. in 2009.

The status of certain *Entamoeba* spp. as either commensals or pathogens has been a subject of debate, particularly regarding *E. dispar* and *E. moshkovskii*, which were previously considered commensals but have been associated with non-invasive infections. In contrast, *E. histolytica* can cause both invasive and non-invasive infections. A new species, *E. bangladeshi*, has also been identified as a cause of diarrhoea in children. However, differentiation between these species based on morphology alone is difficult. Therefore, we conducted a study to provide an update on *Entamoeba* species distribution among diarrhoeal patients. We used a genus-specific primer for the preliminary screening of *Entamoeba* spp. in the first step, followed by a nested primer set based on the 18S rRNA locus to identify each species specifically. This study aims to contribute to the current understanding of the distribution of *Entamoeba* species and their potential pathogenicity.

E. moshkovskii was first identified as a distinct species from Moscow by Tshalaia in 1941. Initially considered to be a free-living environmental strain, it is still a common protozoan species found in anoxic sediments and brackish coastal pools. *E. moshkovskii* is osmotolerant and can be easily cultured in various media suitable for intestinal protozoa, growing at temperatures of 10-15°C and 37°C. In 1961, a strain resembling *E. histolytica* was isolated from a patient who was a resident of Laredo, Texas. The strain named *E. histolytica* Laredo, shares many biological characteristics with *E. moshkovskii*. Later molecular studies confirmed that *E. histolytica* Laredo was, in fact, *E. moshkovskii*, the first human isolate of this species. Nowadays, the significance of other morphologically identical *Entamoeba* species, such as amphizoic *E. moshkovskii*, is increasing, as it has been reported in human patients from various countries. While investigations into the pathogenic potential of *E. moshkovskii* are ongoing, genetic characterization studies from India are limited. Therefore, following the identification of the distribution of *Entamoeba* species, 18S rRNA locus PCR products from *E. moshkovskii*-positive samples were sequenced to further characterize the local isolates. This allowed for the determination of the genetic and phylogenetic structures of the *E. moshkovskii* population. The *E. moshkovskii* population was categorized into various subgroups based on their co-infection status. A haplotype network was constructed to infer the relationship between *E. moshkovskii* haplotypes in different coinfected subgroups. As many organisms can induce diarrhoea, including bacteria, viruses, enteric parasites, and helminths, screening for these diarrheagenic agents was conducted in *E. moshkovskii*-positive diarrhoeal cases. We calculated the statistical

association to evaluate the pathogenic potential of *E. moshkovskii* in diarrhoeal cases solely infected with this species, which are not commonly co-infected with other enteropathogens. This study also investigated the seasonal and geographical distribution of *E. moshkovskii* infection, with a focus on identifying certain SNPs that may correlate with the clinical features of the infection. Overall, this study aimed to investigate the epidemiological importance of *E. moshkovskii* infection in Eastern India and assess its potential as an emerging enteric pathogen in the region. The study findings are expected to provide valuable insights for future research and the development of effective control measures against *E. moshkovskii.*

E. moshkovskii population obtained in this study area was categorized into various subgroups based on their co-infection status. This study also aimed to investigate the parasite genotypes of the diarrhoeal subgroup that was solely infected with *E. moshkovskii* and those co-infected with other diarrhoeagenic pathogens using a multi-locus sequence typing (MLST) approach. In our MLST study, we have incorporated three possible genetic markers associated with diarrhoeal incidence of E. *moshkovskii* infection, namely KERP1, which is a protein-rich in lysine and glutamic acid, amoebapore C (apc), and chitinase. KERP1 is present on the surface of the *E. histolytica* parasite as a trimeric protein complex, and it has the ability to attach to human enterocytes as well as play a role in the development of amoebic liver abscesses (Perdomo et al. 2013). Therefore, KERP1 is considered a crucial virulence factor for *Entamoeba*. The kerp1 gene of *E. moshkovskii* is found to be homologous to that of *E. histolytica*. A study conducted by Weedall in 2020 has revealed that the kerp1 genes of *E. histolytica* EHI_098210 (100% self-match); *E. nuttalli* ENU1_189420 (97% amino acid identity over the whole protein); and *E. moshkovskii* EMO_099600 (45% amino acid identity over part of the protein) are reciprocally the best matches to one another. Amoebapore C (apc) is another gene that plays a role in the virulence of *E. histolytica*. A previous study has discovered that there are SNPs located in the upstream region of the apc gene in *E. histolytica* that are significantly associated with the disease outcomes of Amoebiasis (Das et al. 2021). This Amoebapore C of *E. histolytica* has homologs in other species of *Entamoeba*, such as *E. dispar* strain SAW760 (EDI_206610), *E. invadens* strain IP1 (EIN_133650), and *E. moshkovskii* Laredo (EMO_119370). Therefore, Amoebapore C is considered a potential candidate for our genotyping study. The third gene included in the MLST study is chitinase of *E. moshkovskii*. *Entamoeba* species encode numerous chitinases that possess a conserved type 18 glycohydrolase domain (Vega et al. 1997). During the encystation process of amoeba, chitinase expression is observed (Vega et al., 1997). These chitinase genes contain repetitive

DNA sequences that exhibit significant inter-isolate variability in terms of their repeat types and arrangement patterns in *E. histolytica*. As a potential genetic marker in our MLST study, we selected a chitinase gene from E. *moshokskii* (EMO_056190) that shares homology with *E. histolytica* (KM1_098160). The MLST analysis aimed to identify genotypes statistically associated with the co-infection status of *E. moshkovskii* based on our epidemiological data. Comparative genetic analyses of *E. moshkovskii* populations from various coinfection subgroups have been carried out to identify genetic markers, including single nucleotide polymorphisms (SNPs), significantly associated with the diarrhoeal incidence of *E. moshkovsii* infections. Therefore, it is crucial to explore the relationship between *E. moshkovskii* genotypes and infection status to gain insights into the molecular mechanisms involved in *E. moshkovskii* pathogenesis. Furthermore, it is important to gather genome information of infecting strains from endemic areas worldwide to expand our understanding of this relationship.

E. moshkovskii was originally identified in humans, and there is currently no evidence of its occurrence in farm animals. However, a recent study found the presence of *E. moshkovskii* in non-human primates (NHP), but there is limited data on its prevalence in other animals (Levecke et al. 2010). It is unclear if these animals could serve as a reservoir for the zoonotic transmission of *E. moshkovskii*. Previous studies have focused on detecting cysts or trophozoites in human stool samples using light microscopy or PCR, so the natural host range and distribution of *E. moshkovskii* are largely unknown. Swine husbandry is increasing in India and plays a crucial role in livestock farming. This raises concerns about the potential exposure to zoonotic parasitic agents such as *Entamoeba* spp from pigs. To investigate this, we conducted a study to identify the occurrence of *E. moshkovskii* in stool samples from indigenous pig breeds in and around Kolkata, West Bengal. Our findings suggest that swine may serve as a reservoir for *E. moshkovskii* and could be a possible route for its zoonotic transmission.

In addition to investigating the prevalence, genotyping, and zoonotic potential of *Entamoeba moshkovskii*, this study also focuses on analyzing the high-resolution genotyping of E. histolytica using various polymorphic loci. *E. histolytica* is the main protozoan parasite responsible for amoebiasis, a significant public health issue in India that causes diarrhoeal diseases leading to considerable morbidity and mortality. The factors that determine the differential outcome of *E. histolytica* infection are not yet fully understood, although it is believed that host defense mechanisms and pathogen virulence play primary roles. The specific factors that define the outcome of the disease in the host-parasite relationship are still uncertain.

Over the past two decades, researchers have conducted DNA typing of various polymorphic genetic loci, including serine-rich *E. histolytica* protein (SREHP), Chitinase, and tRNA-linked STR loci (A-L, N-K2, R-R, S-Q, and STGA-D), to investigate a possible link between parasite genotype and the outcome of infection. Several reports have correlated specific genotypes with virulent or avirulent phenotypes. However, a significant limitation of this genotyping strategy is the high divergence of polymorphism in the loci mentioned above, which characterizes several genotypes, even in a limited geographic area (Haghighi et al., 2002). In some studies, the correlation between individual parasite genotypes and different infection outcomes was difficult to evaluate due to a large number of genotypes (Jaiswal et al., 2014). Therefore, researchers recommend characterizing additional isolates using more genetic markers to reveal the mystery behind the genetic polymorphisms in *E. histolytica* and clinical phenotypes of amoebiasis. Among the many molecular markers, SREHP and tRNA-linked array N-K2 are the two highly polymorphic loci that are most important in genotyping studies of E. histolytica infection (Ali et al., 2008). SREHP is a prominent surface antigen that contains repetitive sequences of closely related dodecapeptides and octapeptides. Among the tRNA-linked STR loci, N-K2 is the most polymorphic genetic marker (Das et al., 2014). According to Ali et al. 2007, there was a highly significant difference in the distribution of genotypes between the asymptomatic and liver abscess patient groups of *E. histolytica* at this locus. Loci 1-2 and 5-6 are two tRNA-linked polymorphic loci that contain internal tandem repeats, which were first isolated and characterized by Zaki and Clark in 2000 (Zaki & Clark, 2000). These loci are multicopy and may be arranged in tandem arrays. DNA sequencing of the loci revealed that both contained 6 to 21 copies of 8 to 16 nucleotide repeat units, and changes in the number of repeat units were associated with observed size variation in PCR products (Haghighi et al., 2002). In order to achieve higher resolution in strain typing of *E. histolytica*, multiple loci need to be employed. Thus, we focused on developing a multilocus STR genotyping strategy that is optimal for high-resolution typing (Das et al. 2014). In this study, we genotyped *E. histolytica* clinical isolates obtained from diarrhoeal, non-diarrhoeal, and liver abscess samples using a combination of one polymorphic coding gene (SREHP) and three polymorphic non-coding loci (tRNA linked array N-K2, loci 1-2, and 5-6). Our aim was to investigate the multilocus STR polymorphism and decipher the genetic background of *E. histolytica* isolates that sporadically cause amoebic liver abscesses or become diarrheagenic. Furthermore, we also investigated the genetic pattern and diversity of *E. histolytica* isolates obtained from asymptomatic infected individuals.

Genotyping of *E. histolytica* can be a valuable tool in understanding the drug response of this parasite and informing public health strategies for preventing and treating infections caused by it. This approach has the potential to improve the effectiveness of treatment for amoebic infections caused by *E. histolytica*, leading to better health outcomes for affected individuals. Genotyping of *E. histolytica* based on repetitive regions can enhance our understanding of the diversity and evolution of this intriguing parasite.

As previously mentioned, it is challenging to conduct epidemiological studies on amoebic infections because pathogenic *E. histolytica*, commensal *E. dispar*, and amphizoic *E. moshkovskii* have identical morphological characteristics. In addition, a newly identified species, *E. bangladeshi*, further complicates the identification process. Although there are several molecular methods for identifying *Entamoeba* spp., they tend to be time-consuming, expensive, and require expertise. Moreover, these methods may not be effective for identifying the newly described species *E. bangladeshi*. To address this issue, we developed a single-round multiplex PCR that can simultaneously detect and differentiate *E. histolytica, E. dispar, E. moshkovskii, E. bangladeshi*, and *E. coli*. We also developed a simple and cost-effective PCR-RFLP method for detecting and differentiating *E. histolytica, E. dispar*, and *E. moshkovskii*. We validated both of these methods by screening clinical samples. These strategies could prove to be useful for studying outbreaks of amebiasis both clinically and epidemiologically.

Along with Entamoeba spp., *Cryptosporidium* spp is a major health concern in developing countries like India. The prevalence of *C. hominins* in India is high and consistent across various studies, ranging from 70% to 80% of all typed samples. *Cryptosporidium parvum* is the second most commonly identified species, with a prevalence of 12% to 17%. In addition to these, zoonotic isolates such as *C. felis, C. meleagridis, C. raynae*, and *C. viatorum* have also been identified in India, according to research findings *(*Ajjampur et al. 2007, Das et al. 2019, Xiao & Feng 2008*)*. Diagnosis of cryptosporidiosis typically involves identifying oocysts in stool samples, but this method cannot determine the species of *Cryptosporidium* causing the infection. Since oocysts from multiple species of *Cryptosporidium* are identical in appearance, molecular techniques are essential to accurately identify the species and determine the genotype or subtype responsible for an infection. This is crucial for pinpointing the source and transmission routes of the organism. The 18S rRNA subunit gene is commonly used as a biomarker for PCR-based analysis, but other markers such as HSP70, gp60, ML-1 and 2, HSP70, b-tubulin, COWP, CP-HPS7O and CP-DHFRF have also been used. We performed a sequence analysis of the HSP70 locus in various *Cryptosporidium* isolates collected from

diarrhoeal patients in Kolkata to determine their genotype. The study has the potential to improve epidemiological data and offer valuable insights into efforts aimed at preventing and controlling cryptosporidiosis.

Taking into consideration the aforementioned introductory information, the present study has been divided into seven parts to fulfil its objectives:

- **Chapter III:** Hospital-based surveillance of common diarrheagenic enteric parasites in Kolkata
- **Chapter IV:** Investigating the prevalence of morphologically indistinguishable *Entamoeba* species in diarrhoeal patients.
- **Chapter V:** Genetic characterization of *E. moshkovskii* isolates based on small ribosomal RNA locus.
- **Chapter VI:** Genetic characterization of the *E. moshkovskii* population based on different potential genetic markers.
- **Chapter VII:** Occurrence of *Entamoeba moshkovskii* in pigs with zoonotic potential from eastern India
- **Chapter VIII:** Genotyping of *E. histolytica* isolates based on different polymorphic loci.
- **Chapter IX:** Development of highly specific and sensitive techniques for detection and differentiation of common enteric parasites.

CHAPTER II

REVIEW OF LITERATURE

2.1 Parasitic diarrhoeal disease

Diarrhoea, characterized by loose and watery stool and abdominal pain, is a common symptom of intestinal infections caused by bacteria, viruses, and parasites. Poor hygiene, and contaminated food or water are the primary modes of transmission for these infections. In children under 5, diarrhoeal diseases are the second leading cause of death, resulting in the loss of about 760,000 lives annually (World Health Organization, 2013). Apart from infectious agents, non-infectious medical conditions like lactose intolerance, celiac disease, and pancreatic problems can also cause diarrhoea (Haque et al. 2003).

Different infectious agents, including viruses (rotavirus, enterovirus, Norwalk virus, or hepatitis virus), bacteria (*Escherichia coli*, *Shigella* species, and *Campylobacter jejuni*), and parasites (*Entamoeba histolytica, Giardia lamblia and Cryptosporidium* spp.) can cause diarrhoea (Shah et al. 2009). Some parasitic infections can have a prolonged onset of diarrhoea and last for months, while bacterial and viral infections usually last for 1-2 weeks (Petri, 2003). Parasites are larger and more complex than viruses or bacteria, and their similarity to the host makes them challenging to eradicate. Various parasites like *E. histolytica, G. lamblia, Cryptosporidium* spp.*, Cyclospora cayetanensis*, *Isospora belli*, and *Blastocystis hominis* can cause diarrhoea (Azam et al. 2015). This literature review focuses on diseases caused by commonly occurring enteric parasites, with a specific emphasis on those belonging to the *Entamoeba* genus.

2.2 Amoebiasis

Amoebiasis has a fascinating history that dates back to ancient times. The earliest record of this disease is believed to be from the Sanskrit document Bhrigu-samhita, which was written around 1000 BC (Vaidya & Ray 1982; Cox 2002). The document refers to bloody and mucus diarrhoea, which are common symptoms of amoebiasis. Throughout the middle Ages, "bloody flux" was recorded in Europe, Asia, Persia, and Greece, indicating the widespread prevalence of the disease in those times (Kiple 1993).

The disease was introduced into the New World by Europeans in the 16th century, and it was not until the 19th century that the relationship between dysentery and liver abscess was recognized by James Annersley (Crosby 1986). Budd, 1857 later described the connection between amoebic dysentery and liver abscesses (Cox 2002). In 1875, Losch was the first to discover the amoeba in the stools of dysenteric patients and named them *Amoeba coli*. He also

described the relationship between the parasite and the disease. The cyst form was later described by Quincke and Roos in 1893, and Schaudinn proposed the name *E. histolytica* in 1903. Craig confirmed these findings in 1905, and the pathogenicity of these amoebae was finally proven by the experiments of Walker and Sellards in 1913.

In 1925, Brumpt reported a new species within *E. histolytica*, which he called *Entamoeba dispar*. This species is morphologically similar to *E. histolytica*, but it colonizes the human gut without any invasive potential. The recent acceptance of *E. dispar* as a distinct but closely related protozoan species has had significant implications for the epidemiology of amoebiasis, as most asymptomatic infections found worldwide are now attributed to this non-invasive amoeba (Clark 1998).

E. moshkovskii, which is closely related to *E. histolytica* and *E. dispar*, appears identical to them under a microscope in both its cyst and trophozoite forms. Initially believed to be a common free-living protozoan species in anoxic sediments and environments such as brackish coastal pools, this species of *Entamoeba* was first discovered in sewage in Moscow by Tshalaria in 1941 (Scaglia et al. 1983). The first human case of *E. moshkovskii* was found in 1961 in a person from Laredo, Texas, who had symptoms such as diarrhoea, weight loss, and epigastric pain (Dreyer 1961). These findings suggest that *E. moshkovskii* may also have pathogenic potential (Shimokawa et al. 2012).

E. bangladeshi is another species of amoeba that is closely related to *E. histolytica*, *E. dispar* and *E. moshkovskii.* It was first identified in Bangladesh. It was first described in 2002 by a group of researchers from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and the University of Virginia, USA (Clark et al. 2007). In their study, the researchers collected faecal samples from patients with diarrhoea in Bangladesh and examined them for amoebic infections. They discovered a new species of *Entamoeba* that had not been previously described. The researchers named the new species *E. bangladeshi* based on its discovery in Bangladesh. Since its discovery, there have been further studies on *E. bangladeshi* to understand its biology and pathogenicity. It is now known to be a relatively common species of *Entamoeba* in Bangladesh and other parts of the world, and it has been associated with gastrointestinal infections.

2.2.1 *E. histolytica*

2.2.1.1 Taxonomic positions

The original classification of true amoebae found in humans was established by the Committee on Systematics and Evolution of the Society of Protozoologists in 1980, placing them in the family Endamoebidae, order Amoebida, subclass Gymnamoebia, class Lobosea, superclass Rhizopoda, subphylum Sarcodina, and phylum Sarcomastigophora (Levine et al 1980). However, recent advancements in our knowledge of the morphological ultrastructure, chemical and physical properties of biological macromolecules of these parasites have rendered this classification of the protozoa obsolete. Therefore, a new taxonomic scheme is required (Cox 1992; Corliss 1994). According to Corliss's revised classification, all amoebae found in humans are now placed in the phylum Rhizopoda, class Entamoebidea, and are further classified in the order Endamoebida and the family Endamoebidae.

2.2.1.2 Morphology and life cycle

A. Trophozoite

Trophozoites, the active form of *E. histolytica*, can range in size from 12 to 60 µm in diameter, with larger forms found in tissue and smaller ones in non-dysenteric stools or cultures, measuring 7 to 30 µm (Guerrant, 1986). The ectoplasm appears transparent, while the endoplasm is granular and may contain bacteria and/or erythrocytes in various stages of digestion. The trophozoite nucleus is spherical and varies from 4 to 7 µm. In stained preparations, the nucleus displays a delicate nuclear membrane lined with a single layer of uniformly distributed fine chromatin granules (Dobell, 1919), a small central karyosome surrounded by a clear halo, and a fine thread of a spoke-like radial arrangement of "linin" network traversing the space between the karyosome and the nuclear membrane. Initially, *E. histolytica* was thought to lack cytochrome, classical Embden-Meyerhoff pathway, rough endoplasmic reticulum, and Golgi apparatus. However, further studies revealed the presence of smooth endoplasmic reticulum and Golgi-like elements in this organism (Chavez et al. 1978). Although *E. histolytica* does not possess structures resembling mitochondria, research shows the presence of a residual organelle called "mitosome" or "crypton" in the cytoplasm, discovered through immunolocalization (Tovar et al. 1999; Mai et al. 1999). While cytoskeleton or integrins have not been clearly defined in *E. histolytica*, some reports suggest the presence of cytoplasmic microfilaments, nuclear microtubules, and myosin (Vargas et al. 1997), and the description of a b1 integrin-like molecule (Talamas-Rohana et al. 1998; Flores-

Robles et al. 2003). Additionally, ribosomes form crystalline arrays in the cytoplasm of the trophozoite (Rosenbaum & Wittner, 1970).

B. Pre-cyst

During the pre-cyst stage, the trophozoite undergoes a transformation to become roughly the same size as the cyst. The cytoplasm is purged of all nutrient inclusions, but may still contain scattered glycogen deposits and occasional chromatoid bodies, which consist of ribosomes (Proctor & Gregory, 1973). The pre-cystic form is characterized by a single nucleus that becomes enlarged, and its karyosome is typically located off-center.

C. Cyst

The cyst stage is characterized by a round or slightly oval shape, with a size ranging between 8 to 14 μm. Chemical analysis and X-ray diffraction studies of purified preparations have shown that the cyst wall contains chitin (Arroyo-Begovich et al. 1980). The nucleus consists of one to four distinct units, with the nuclear membrane uniformly surrounded by peripheral chromatin. The karyosome is small and usually centrally positioned within the nucleus. Polyribosomes and vacuoles filled with dense fibrogranular materials are closely juxtaposed to the cytoplasmic side of the plasma membrane (Chavez et al. 1978). Generally, glycogen and chromatoidal material disappear as the cyst matures.

D. Metacyst

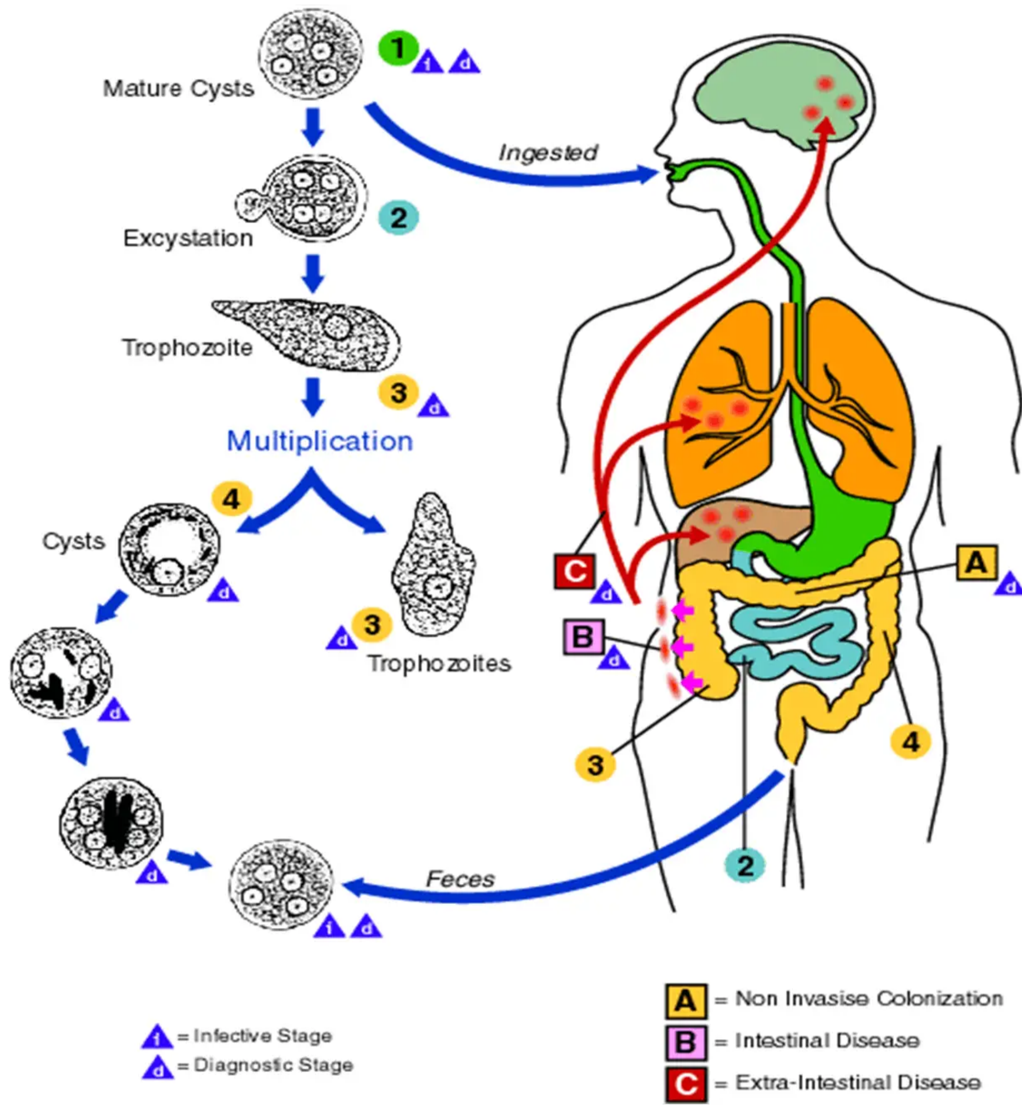
During excystation, the encysted amoeba becomes highly active, detaching itself from the cyst wall. The quadrinucleate amoeba escapes through a small pore in the cyst wall, and the nuclei tend to aggregate together (Bruckner, 1992). This stage is referred to as the metacyst.

F. Life cycle:

E. histolytica, a parasite that causes amoebic dysentery, has a complex life cycle that begins with the ingestion of contaminated food or water, or through oral-anal sexual practices. The infective stage is the cyst form, which is highly resistant to chlorination, gastric acidity, and desiccation, and can survive for several weeks in moist environments. Animal studies show that as few as 1-100 cysts can cause disease, which is comparable to the infectious dose of *Shigella* sp.

Once ingested, the cyst passes through the stomach and reaches the small bowel, where it undergoes excystation, a process that involves nuclear and cytoplasmic division. This results in the formation of eight trophozoites, which migrate to the large bowel. The trophozoites multiply by binary fission and can form new cysts by aggregating in the intestinal mucin layer, leading to a self-limited and asymptomatic infection. However, they can also invade the colonic epithelium, leading to inflammation and destruction of the bowel wall. Factors that determine whether invasion or asymptomatic colonization occurs are not fully understood but may include differences in *E. histolytica* genotypes, host genetic variation, gut microbiome, nutritional status, and immunocompetence.

Following the invasion, the trophozoites can spread to the liver through hematogenous dissemination via the hepatic portal circulation. In rare cases, they can cause extra-abdominal involvement of the CNS, pulmonary, or cardiac organs via hematogenous dissemination or direct extension from a liver abscess. The life cycle of *E. histolytica* is completed when trophozoites undergo encystation and are passed out of the body in faeces into the environment. Trophozoites passed in stool cannot survive for long outside the body (**Fig 2.1**).



Life Cycle of *Entamoeba histolytica*
(Source: CDC)

Fig 2.1 Life cycle of *E. histolytica.*

2.2.1.3. Biochemistry and metabolism

The metabolic characteristics of *E. histolytica* show a closer resemblance to microaerophilic and anaerobic bacteria, rather than typical eukaryotes (Fahey *et al.* 1984). This could be attributed to the evolutionary processes of secondary gene loss and lateral gene transfer (LGT), predominantly from bacterial lineages, that have shaped the metabolism of *E. histolytica.*

A. Carbohydrate metabolism

E. histolytica lacks true mitochondria and instead possesses mitosomes, which do not participate in energy production or redox balance maintenance. Thus, *E. histolytica* solely relies on substrate-level phosphorylation for energy generation. This protozoan is an obligate fermenter that utilizes bacterial-like fermentation enzymes and lacks enzymes linked to the mitochondrial electron transport chain or TCA cycle, according to (Loftus et al. 2005). Glucose is the primary energy source for the trophozoite stage of *E. histolytica* which is the only stage that has been investigated in vitro. However, recent studies suggest that amino acids might also have a significant role in energy metabolism (Anderson and Loftus, 2005). Energy metabolism of *E. histolytica* is confined to the cytosol, where glucose is converted anaerobically to acetate, ethanol, and CO2 (Coombs and Müller, 1995). *E. histolytica* utilizes the classical Embden-Meyerhoff glycolytic pathway, but with a unique modification in which glycolytic enzymes rely on inorganic pyrophosphate (PPi) instead of ATP for catalysis (Mertens, 1993). During the trophozoite stage, the PPi-dependent PFK (step 3b) is the primary route for glycolytic flux, although the ATP-dependent enzyme (step 3a) is also present (Chi et al. 2001). The parasite employs two distinct enzymes to produce pyruvate from phosphoenolpyruvate (PEP): pyruvate kinase (PYK, step 12a) which generates ATP from ADP, and pyruvate phosphate dikinase (PPDK, a PPi-dependent enzyme, step 12b) which produces ATP from AMP with the contribution of PPi. However, research by (Saavedra-Lira et al. 1998 and Moreno-Sanchez et al. 2008) suggests that the contribution of PYK is minimal in *E. histolytica*. *E. histolytica* uses PEP carboxytransphosphorylase, a PPi-dependent enzyme (step 13b), instead of the typical PEP carboxykinase (step 13a) enzyme. Glycolytic pathway of the parasite is extended and takes place in the cytosol. Pyruvate is converted to acetyl-CoA by pyruvate: ferredoxin oxidoreductase (PFOR, step 16a), where ferredoxin accepts electrons produced by PFOR and ferredoxin:NAD oxidoreductase (step 17) transfers them to NAD+. Acetyl-CoA can be transformed into acetate with the aid of 'ADP-forming' acetyl-CoA synthetase (AceCS), which

generates ATP (step 19a). Alternatively, it can be reduced to ethanol, regenerating NAD+ (step 22) (Reeves, 1984) (see **Fig 2.2**).

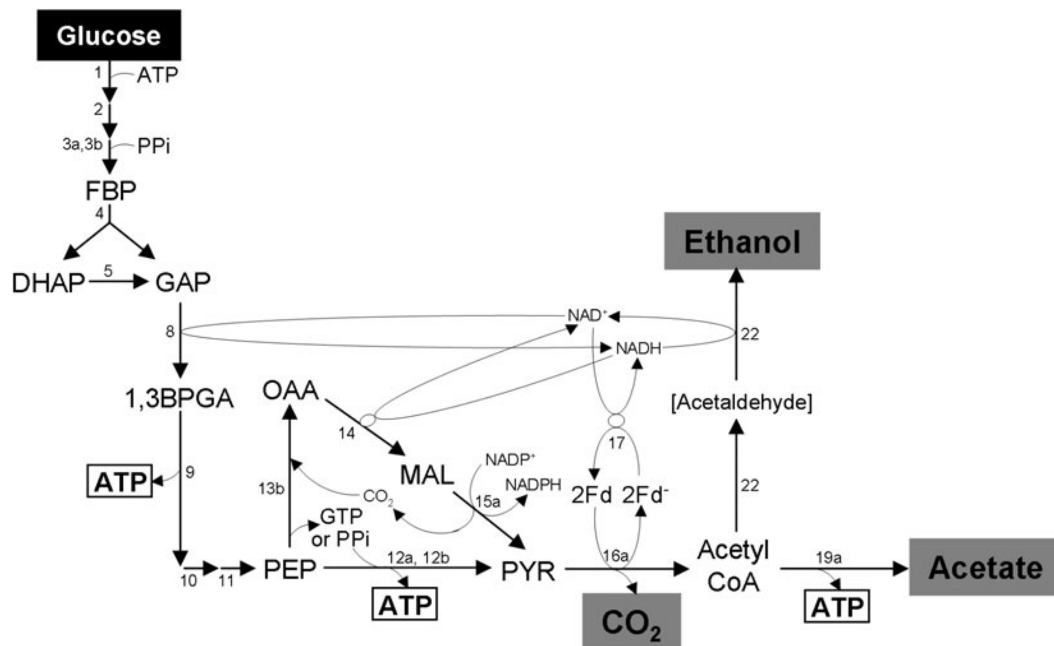


Fig 2.2 Carbohydrate metabolism in the trophozoite stage of *Entamoeba histolytica* (adapted from Köhler and Voigt, 1988; Coombs and Mu¨ller, 1995) (Bringaud et al. 2009). Excreted end products of glucose metabolism are in black characters on a grey background. The enzyme-bound metabolite is indicated by brackets (acetaldehyde). ATP molecules produced by substrate-level phosphorylation are boxed.

Abbreviations: PPi, inorganic pyrophosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 1,3BPGA, 1,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MAL, malate; PYR, pyruvate; Fd, ferredoxin.

Enzymes are: 1, hexokinase: 2, glucose-6-phosphate isomerase; 3a, ATP-dependent phosphofructokinase (ATP-PFK); 3b, PPi-dependent phosphofructokinase (PPi-PFK); 4, aldolase; 5, triose-phosphate isomerase; 8, glyceraldehyde-3-phosphate dehydrogenase; 9, phosphoglycerate kinase; 10, phosphoglycerate mutase; 11, enolase; 12a, pyruvate kinase (PYK); 12b, pyruvate phosphate dikinase (PPDK); 13b, phosphoenolpyruvate carboxytransphosphorylase; 14, malate dehydrogenase; 15a, malic enzyme; 16a, pyruvate:ferredoxin oxidoreductase; 17, ferredoxin:NAD oxidoreductase; 19a, Acetyl-CoA synthetase 'ADP forming' ('ADP forming' AceCS); 19b, acetate:succinate CoA-transferase (ASCT); 20, succinyl-CoA synthetase (SCoAS); 22, bifunctional acetyl-CoA reductase/alcool dehydrogenase.

(Source: Bringuad et al. 2009, Doi: https://doi.org/10.1017/S0031182009991843)

B. Energy storage

Trophozoites contain a large number of glycogen granules in their cytoplasm, which were first identified through electron microscopy and later studied biochemically (Rosenbaum and Wittner 1970, Takeuchi *et al.* 1977). These granules are associated with an enzyme called glycogen phosphorylase, which breaks down the linear chains of various glucopolysaccharides into glucose 1-phosphate and orthophosphate (Werries & Thurn, 1989). However, glycogen phosphorylase can only break down the linear chains down to the a-1,6 branch points, leaving behind a core molecule called limit dextrin. For further degradation of limit dextrin, a debranching enzyme that exhibits both amylo-1,6-glucosidase and 4-a-glucanotransferase activities has been purified (Werries et al. 1990). Recently, Wu and Müller, 2003 cloned two glycogen phosphorylase genes (Wu and Müller, 2003).

Before entering the glycolytic pathway, glucose 1-phosphate undergoes isomerization to glucose 6-phosphate, which is facilitated by an enzyme called phosphoglucomutase. The glycogen precursor UDP-glucose is synthesized from UTP and glucose 1-phosphate by an enzyme called UTP:glucose-1-phosphate uridylyltransferase. Two types of UTP hexose-1-phosphate uridyltransferases have been identified through biochemical characterization: a larger enzyme of 45 kDa that specifically acts on glucose 1-phosphate, and a less specific enzyme of 40 kDa that can use both galactose 1-phosphate and glucose 1-phosphate as substrates (Lobelle-Rich and Reeves, 1983).

C. Electron transport

Entamoeba consumes oxygen without producing peroxide when growing in microaerophilic conditions, although it is not fully clear if oxygen is the final electron acceptor during pyruvate oxidation. The process of electron transfer to NAD under anaerobic conditions is also not well understood. Based on experimental evidence, it is suggested that in the absence of heme iron-containing cytochromes, enzymes such as soluble NADPH diaphorase and/or NADPH:flavin oxidoreductase could transfer electrons released during pyruvate oxidation to ferredoxin's iron-sulfur centers using one or more flavins like FMN. The subsequent steps of electron transfer after ferredoxin are not known (Reeves, 1984; McLaughlin & Aley 1985; Loftus et al. 2005). All of these enzymes are found in the soluble fraction of *Entamoeba* extracts, which indicates that electron transfer is not compartmentalized. (Reeves 1984; McLaughlin and Aley 1985; Loftus et al. 2005).

D. Amino acid catabolism

E. histolytica is capable of utilizing amino acids not just for uptake, but also for energy production (Reeves 1984; Zuo and Coombs 1995). This is particularly significant in anaerobic conditions where glycolysis can only generate a limited amount of ATP from glucose. Interestingly, the genome of *E. histolytica* contains several genes that code for enzymes involved in the catabolism of amino acids, many of which have bacterial origins (Anderson and Loftus 2005). The degradation of amino acids typically begins with a transamination reaction that produces a 2-ketoacid. *E. histolytica* has five ORFs that have been identified as aminotransferases. Pyruvate is a common intermediate in the catabolism of amino acids, and can be produced from the degradation of various 2-ketoacids.

Aspartate and asparagine

In the presence or absence of glucose, *E. histolytica* is capable of uptaking both asparagine and aspartate (Zuo and Coombs 1995). The genome of this organism contains four putative asparaginases, out of which three are identical, while the fourth shares only 48% amino acid identity with the others. Asparaginase catalyzes the conversion of asparagine to aspartate, while simultaneously releasing ammonia.

Serine, threonine

In the presence or absence of glucose, *E. histolytica* takes up both serine and threonine (Zuo and Coombs 1995). Serine can be converted to pyruvate and ammonia by the pyridoxal phosphate-dependent enzyme serine dehydratase (L-serine ammonia-lyase). Takeuchi et al. characterized this enzyme and demonstrated that the addition of serine to the culture medium resulted in an increase in oxygen consumption (Takeuchi et al. 1979). Similarly, threonine can be broken down to 2-oxobutanoate by threonine dehydratase (threonine ammonia-lyase) in a similar reaction. Both ketoacids, 2-oxobutanoate and pyruvate, can be oxidized to acetyl-CoA or propionyl-CoA by PFOR. It has been shown that these catabolic reactions can be catalyzed by the same enzyme, as seen in yeast, for example (Ramos and Wiame, 1982).

Methionine, homocysteine and cysteine

E. histolytica has two types of methionine g-lyase enzymes called EhMGL1 and EhMGL2, which have similar molecular weights but differ significantly in their substrate specificity,

isoelectric point, and biochemical properties (Tokoro et al. 2003). Both enzymes break down methionine into methanethiol, ammonia, and 2-oxobutanoate, but they have distinct enzymological characteristics (Tokoro et al. 2003).

Arginine

The arginine deiminase pathway plays a crucial role in energy generation by producing one ATP molecule through the breakdown of arginine to ornithine in *G. intestinalis* and *T. vaginalis* (Knodler et al. 1994; Linstead and Cranshaw 1983; Schofield and Edwards 1994). However, the genome of *E. histolytica* does not contain any arginine deiminase gene or dihydrolase pathway. Instead, *E. histolytica* degrades arginine either through arginase to form ornithine or through arginine decarboxylase to produce agmatine, a reaction that consumes protons and may aid in acid resistance for the passage of cysts through the human stomach (Anderson and Loftus 2005). The degradation of arginine also appears to prevent NO synthesis and amoebicidal activity by depleting arginine as a substrate for human macrophages, suggesting an additional function for arginine metabolism in *E. histolytica* (Elnekave et al. 2003).

Glutamate, glutamine

Aerobic organisms typically utilize the 2-oxoglutarate produced from glutamate via transaminase activity as a substrate in the citric acid cycle for further breakdown. However, in *E. histolytica*, which also possesses transaminases, the 2-oxoglutarate molecule can undergo oxidation by PFOR to generate succinyl-CoA, resulting in the production of one molecule of ATP (Clark et al. 2007). *E. histolytica* contains several other gene products that may also be involved in the metabolism of glutamine and glutamate. Notably, the organism lacks a glutaminase enzyme, which is typically responsible for catalyzing the hydrolysis of glutamine (Clark et al. 2007).

Tryptophan

The enzyme tryptophanase, which relies on pyridoxal 5'-phosphate (PLP), can degrade tryptophan into indole, pyruvate, and ammonia. There is only one potential gene identified for this enzyme (Clark et al. 2007). Tryptophanase has been discovered exclusively in bacteria and *T. vaginalis*, and it is also considered a possible candidate for lateral gene transfer (LGT).

Alanine

Although it is possible for alanine to be converted to pyruvate by alanine aminotransferase, it appears that this enzyme is not utilized by *E. histolytica* under the tested culture conditions, as the organism has been observed to excrete alanine (Zuo and Coombs 1995). This excretion process may serve the purpose of eliminating excess nitrogen from the cell when a functional urea cycle is absent.

Other amino acids

E. histolytica lacks most of the enzymes involved in the metabolism of branched-chain amino acids. However, it has a potential branched-chain amino acid aminotransferase enzyme that can convert leucine, isoleucine, and valine into 2-oxoisocaproate, 2-oxo-3-methyl valerate, and 2-oxovalerate, respectively (Clark et al. 2007). This process may either generate ammonia or transfer the amino group to 2-oxoglutarate to form glutamate (Clark et al. 2007).

The fate of certain amino acids such as glycine, proline, phenylalanine, tyrosine, and lysine in *E. histolytica* remains poorly understood at present (Clark et al. 2007).

D. Polyamine metabolism

The absence of enzymes such as S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), spermidine synthase (EC 2.5.1.16), and spermine synthase (EC 2.5.1.22) in *E. histolytica* indicates that this parasite likely does not have polyamine metabolism (Anderson and Loftus 2005). However, *E. histolytica* does have genes for arginase and arginine decarboxylase, which could play a role in producing putrescine either through agmatine and agmatinase (EC 3.5.3.11), or through ornithine and ornithine decarboxylase (EC 4.1.1.17). The NMR spectroscopy results showing a high concentration of putrescine (9.5 mM) in trophozoites highlights the importance of putrescine in the physiological processes of *E. histolytica* (Bakker-Grunwald et al. 1995). However, the destiny of putrescine remains uncertain since the presence of neither spermine nor spermidine has been established in this organism. The existence of trypanothione, a thiol-containing spermidine, in *E. histolytica* is a subject of debate among researchers (Ondarza et al. 1997; Ariyanayagam and Fairlamb 1999). Trypanothione is an essential thiol in trypanosomes and leishmania, composed of two glutathione molecules linked by a spermidine linker (Fairlamb and Cerami 1992). Initially, there were reports suggesting the presence of trypanothione in *E. histolytica*, but later research contradicted this claim. However,

recent studies have provided evidence supporting its existence in the organism. Nevertheless, the gene responsible for encoding trypanothione reductase in *E. histolytica* strain HK-9 (AF503571) does not have any homolog in the HM-1:IMSS genome. Although there is ongoing debate about the presence of trypanothione in *E. histolytica*, most researchers agree that cysteine is the main thiol in the organism (Fahey et al. 1984).

E. histolytica possesses two types of ornithine decarboxylases, one of which is a 46 kDa enzyme that resembles enzymes found in plants and vertebrates, and the other is a 96 kDa enzyme that can decarboxylate ornithine, arginine, and lysine. The 46 kDa enzyme has been characterized biochemically and has been found to be resistant to difluoromethylornithine (DFMO), which is also true for *E. histolytica* (Arteaga-Nieto et al. 2002; Gillin et al. 1984). Although arginine decarboxylase is present in *E. histolytica*, the organism does not seem to have agmatinase, which is necessary for converting agmatine to putrescine and urea. The function of arginine decarboxylase in *E. histolytica* is not well understood, but it may play a role in acid resistance (Clark et al. 2007).

E. Biosynthesis of amino acids

Cysteine and serine

A significant reduction in metabolism can be observed in the biosynthesis of amino acids, as evidenced in several organisms. For instance, in *E. histolytica*, biosynthetic pathways for most amino acids, with the exception of serine and cysteine, have been lost (Ali et al. 2003; Clark et al. 2007). Likewise, *P. falciparum*, which primarily relies on acquiring amino acids from host haemoglobins, does not possess the ability to biosynthesize most amino acids (Gardner et al. 2002). *E. histolytica* shows remarkably high intracellular concentrations of certain amino acids, namely glutamate, leucine, valine, and proline, in descending order of abundance (Bakker-Grunwald et al. 1995). The concentrations of glutamate and proline, in particular, are significantly higher in the cells compared to the growth medium, at 21 mM and 7.3 mM respectively, as opposed to 5.9 mM and 1.8 mM in the medium (Bakker-Grunwald et al. 1995). Notably, glutamate constitutes more than one-third of the total amino acid pool, indicating its probable central role in the homeostasis of amino acids and energy metabolism in general. Thus, it is probable that the high intracellular concentrations of specific amino acids, such as glutamate, leucine, valine, and proline, in *E. histolytica*, are due to active uptake facilitated by unidentified amino acid transporters. Interestingly, despite the loss of most other amino acid biosynthetic pathways, the biosynthesis of serine and cysteine pathways has been preserved in

this parasite. This preservation is likely due to the essential physiological role of cysteine, which serves as the primary intracellular thiol in *E. histolytica*. The cysteine biosynthetic pathway consists of two main steps: first, serine acetyltransferase produces O-acetylserine by combining serine and acetyl-coenzyme A, followed by cysteine synthase, which transfers an alanyl group from O-acetylserine to sulphide, leading to the formation of cysteine. *E. histolytica* possesses three genes each for cysteine synthase and serine acetyltransferase. Cysteine synthases 1 and 2 are considered to be allelic isotypes, while cysteine synthase 3 is distinct, sharing only 83% identity with cysteine synthases 1 and 2 (Nozaki et al 1998b). The crystal structure of O-acetylserine sulfhydrylase has recently been unveiled, highlighting its crucial role in the de novo synthesis of L-cysteine (Chinthalapudi et al. 2008). Notably, the *E. histolytica* phosphoserine aminotransferase L-cysteine exhibits close kinship with PSAT from bacterioids, suggesting that *E. histolytica* may have acquired this gene from bacterioids through lateral gene transfer (Ali and Nozaki, 2006).

F. Lipid metabolism

E. histolytica is unable to utilize the high energy content of lipids like fatty acids due to the absence of oxidative phosphorylation. As a result, phospholipids and cholesterol primarily serve as membrane components in this organism. These lipids are predominantly obtained from the host or food sources, although *E. histolytica* does possess the ability to synthesize, modify, and attach lipids to proteins to some extent (Das et al. 2002; Sawyer et al. 1967).

Table 2.1 presents certain lipid functions analyzed in amoebae that share similarities with those observed in other eukaryotes, but also display distinct characteristics specific to *E. histolytica* (Laughlin et al. 2004; Powell et al. 2006; Byekova et al. 2010; Coskun and Simons 2011).

The trophozoites of *E. histolytica* are able to synthesize phosphatidylcholine and phosphatidylethanolamine using the Kennedy pathway, while sphingolipids, phosphatidylserine, and phosphatidylinositol are produced via processes similar to those used by other eukaryotes (**Fig 2.3**). However, these organisms lack the necessary enzymes for synthesizing cholesterol and fatty acids, and therefore must scavenge them from the host or culture medium using specific mechanisms. For instance, cholesterol, a critical molecule for the expression of virulence, is transported into the trophozoites from the medium via the EhNPC1 and EhNPC2 proteins. Once inside the cell, lipids are distributed through various pathways, including the participation of the endosomal sorting complex required for transport (ESCRT), which is involved in vesicle fusion and fission. Cholesterol interacts with

lysobisphosphatidic acid (LBPA), a phospholipid, and with EhADH, an ALIX family protein also involved in phagocytosis (Castellanos-Castro et al. 2020).

It is possible for *E. histolytica* to produce a significant amount of the glycerophospholipids it needs (**Fig 2.3**). Furthermore, the parasite may utilize molecular recycling as a means of obtaining materials for the constant remodelling of its membranes, which occurs during various processes, both related and unrelated to the virulence mechanism of trophozoites.

Table: 2.1 Lipids in *E. histolytica*.

Lipid	Abundance[a]	Abundance[b]	Function	References
PE	14	19	Donor of polar heads to glycoconjugate lipoproteins. Vesicles fusion and fission	Wong-Baeza et al., 2010 Vance 2015
PS	9	8	Signaling Binding ESCRT to form ILVs It is associated with EhCaBP3 protein during phagocytosis	Avalos-Padilla et al., 2018 Aslam et al., 2012
PC	27	48	Membrane composition. Precursor of lytic molecules	Weltzien, 1979
PA	nr	nr	Binding phosphatidylcholine transfer protein-like (EhPCTP-I)	Piña-Vázquez et al., 2014
LBPA	nr	nr	Acidic vesicular trafficking	Castellanos-Castro et al., 2016
PI	7	5	Binding ESCRT Precursor of phosphoinositides	Avalos-Padilla et al., 2018 Sharma et al., 2019
PI(3,4,5)P_3	nr	nr	Phagocytosis Locomotion	Ghosh et al., 1999 Meza, 2000
PI(4,5)P_2	nr	nr	Pinocytosis and adhesion Present in lipid rafts, may regulate trophozoite motility Binds to cytoskeleton and maintain the cell shape and integrity	Ghosh et al., 1999 Koushik et al., 2013 Sharma et al., 2019
PI3P	nr	nr	Phagosomal cup formation	Nakada-Tsukui et al., 2009 Powell et al., 2006
SL	41	17	Lipid rafts formation Cellular adherence and fluid endocytosis	Goldston et al., 2012
SM	3	traces	Forming ceramides in parasite membranes	Mfotie Njoya et al., 2014
CAEP	10	16	Membrane stability	Cerbón and Flores, 1981
CEP	28	nr	Cytotoxicity protection	Cerbón and Flores, 1981
Unidentified	nr	9	nr	Aley et al., 1980
Cholesterol	nr	46*	Membrane composition Virulence properties	Goldston et al., 2012

Relative abundance is given as % total lipid phosphorous.
[a]*Cerbón and Flores (1981).*
[b]*Aley et al. (1980).*
**Molar ratio cholesterol/mol of phospholipid, nr, Not reported.*

(Source: Castellanos-Castro et al. 2020, DOI: 10.3389/fcimb.2020.00075)

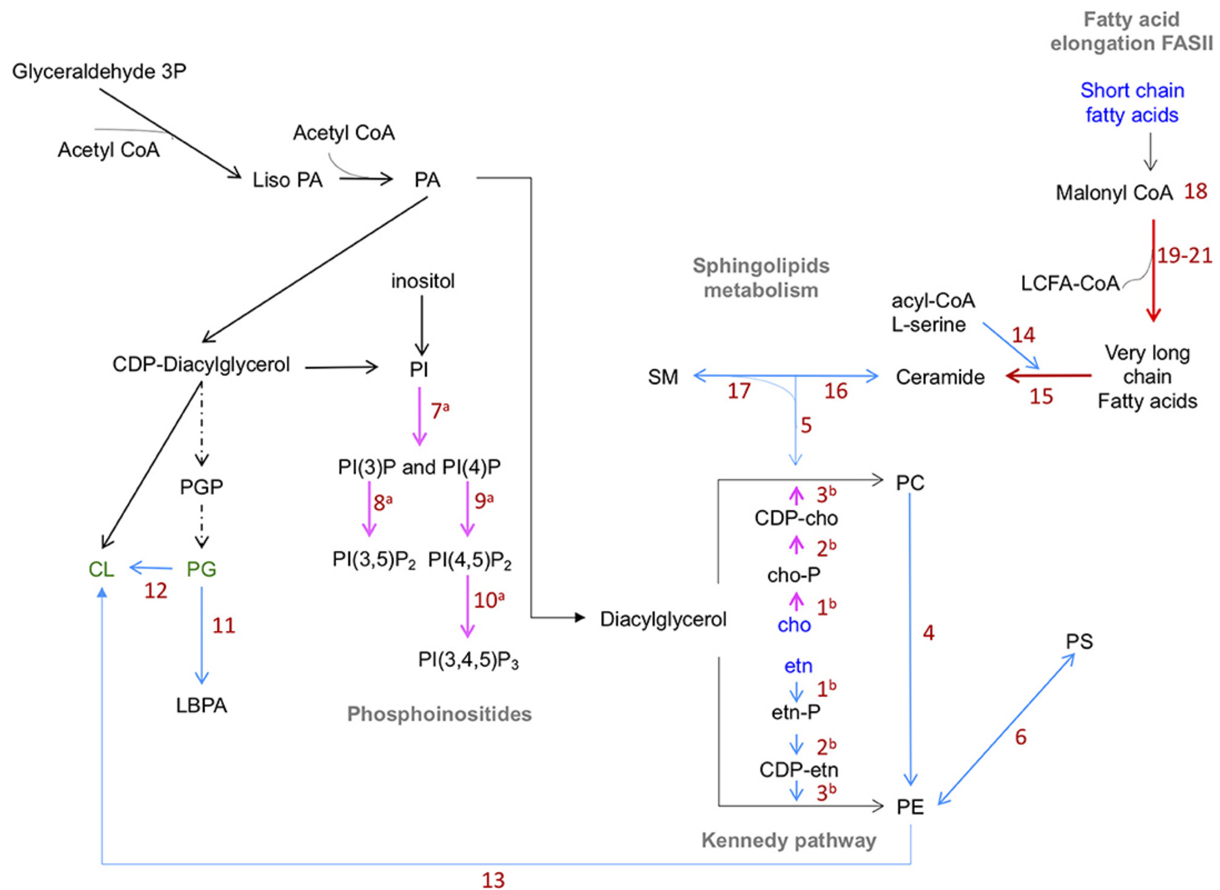


Fig 2.3 Predicted lipid synthesis pathways of phospholipids in *E. histolytica*.
Blue lines: steps catalyzed by enzymes reported in KEGG database. Red lines: enzymes found by proteomics or transcriptomics analyses with inferred function. Pink lines: enzymes previously reported. Dotted black lines: enzymes not found in genome database. Abbreviations in blue: compounds up taken from the medium. Abbreviations in green: phospholipids not yet identified. PE biosynthesis: ethanolamine is phosphorylated by ethanolamine kinases (1) and activated by ethanolamine phosphate cytidyl-transferase (2). CDP-ethanolamine is transferred to diacylglycerol by ethanolamine phosphotransferase (3) to produce PC and PE. PE, methylated by phosphoethanolamine N-methyltransferase (4), produce PC. SM, catabolized by sphingomyelinases (SMA) (5), produce phosphocholine, which follows the Kennedy pathway. PS is synthesized from PE by PS synthase 2 (6). PI(4)P and PI(3)P are produced from IP by the action of type III EhPI(4)K and EhPI3K respectively (7). PI(3,5)P_2 is generated from PI(3)P by the action of EhPIPKIII (8). PI(4,5)P_2 is produced from PI(4)P by EhPIPKI (9). EhPI3KC1 (10) produces PI(3,4,5)P_3 from PI(4,5)P_2. Enzymes involved in the production of PG were not detected in *E. histolytica* genome. PL, AT, and TA enzymes (11) produce LBPA from PG. Cardiolipin is produced from PG or PE by synthase CMP-forming (12) and phospholipase D respectively (13). In sphingolipid metabolism, the enzymes involved are SPT (14), Cer S and its orthologs LAG family proteins (15) are involved in the production of ceramides, which can be transformed in complex SL by SL synthase (16). ASM participate in the ceramide's synthesis by the hydrolysis SM (17). Enzymes involved in fatty acid synthesis pathway were not detected in amoeba genome. Four putative enzymes that participate in the fatty acid elongation cycle: 3-ketoacyl-CoA (18), very-long-chain 3-oxoacyl CoA reductase (19), very-long-chain-3-hydroxyl-CoA dehydratase (20), and very-long-chain enoyl-CoA reductase (21). AcetylCoA, Acetyl coenzyme A; PA, Phosphatidic acid; etn, ethanolamine; CDP-, Cytidine diphosphate; cho, choline; -P, phosphorous; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; (PI(4,5)P_2, PI(3,5)P_2), phosphatidylinositol bi-phosphate; (PI(3,4,5)P_3), phosphatidylinositol (3,4,5) tri-phosphate; CL, cardiolipin; LBPA, lysobisphosphatidic acid; SM, sphingomyelin; PIPK, phosphatidylinositol phosphate kinases; AT, acyltransferases; TA, transacylases; PL, phospholipases; SPT, serine palmitoyl transferase; Cer S, ceramide synthase; LAG, longevity-assurance; ASM, acid sphingomyelinases.

(Source: Castellanos-Castro et al. 2020, DOI: 10.3389/fcimb.2020.00075)

G. Nucleic acid metabolism

E. histolytica, like many other protistan parasites, is incapable of de novo synthesis of purines (Reeves 1984). Analysis of its genome indicates that its nucleic acid metabolism is comparable to that of other lumenal parasites, such as *G. intestinalis* and *T. vaginalis*, which also lack pyrimidine synthesis and thymidylate synthase (Aldritt et al. 1985; Wang and Cheng 1984). Furthermore, *E. histolytica* shares with *G. intestinalis* the feature of lacking ribonucleotide reductase (Baum et al. 1989). The presence of ribonucleotide reductase in the genomic sequences of *E. invadens* and *E. moshkovskii* indicates that this enzyme was recently lost or replaced in *E. histolytica*. This loss of nucleic acid metabolism capability is a rare occurrence among eukaryotes, and it may have happened as a result of an adaptation of the parasite to living in an organic nutrient-rich environment. *E. histolytica* has two distinct 28 kDa nucleases that are released during in-vitro cultivation. It is suggested that these nucleases may function at a distance from the parasite to acquire nucleosides from the host, which are vital for meeting the essential purine and pyrimidine requirements of E. *histolytica* (McGugan et al. 2007).

2.2.1.4. The Genome

Entamoeba is a genus of parasites that infect a wide range of hosts, and species differentiation has traditionally been based on simple morphological features, such as the number of nuclei per cyst. However, this approach has been challenged by the fact that some species, like *E. gingivalis*, do not form cysts. The advent of genome sequencing has revolutionized the concept of taxonomic discrimination between *Entamoeba* species. For example, the *E. histolytica* genome project, which was launched in 2000 with funding from the Wellcome Trust and the National Institute of Allergy and Infectious Diseases (NIAID), has been a collaborative effort between the Wellcome Trust Sanger Institute and The Institute for Genomic Research (TIGR) in the UK and the USA, respectively. In 2005, the complete genome of *E. histolytica* was published and analyzed in draft form. Since then, the genome has undergone further re-assembly and re-annotation (Loftus et al. 2005; Lorenzi et al. 2010; Clark et al. 2007). The *Entamoeba* genome database can be accessed at http://pathema.tigr.org/pathema/Entameba_resources.html. Pathema, which can be accessed at http://pathema.jcvi.org, is one of the eight Bioinformatics Resource Centers (BRCs) established and funded by the National Institute of Allergy and Infectious Diseases (NIAID). Its purpose is to provide a central resource for the bio-defence and infectious disease research

community. Among its many resources, Pathema also includes information about the genome sequences of *E. histolytica* (Brinkac 2009).

A. Karyotype and chromosome structure

The estimated size of the current *E. histolytica* genome assembly is approximately 23.7 million base pairs (Mbp). According to pulse-field gel analysis, there are 14 chromosomes with sizes ranging from 0.3 to 2.2 Mbp, and there may be a ploidy of 4 (Willhoeft and Tannich, 1999). However, the genome composition of *E. histolytica* has been found to be diverse, as assessed in *Entamoeba* cultures grown in isolation (Lohia 2007).

B. Whole genome sequencing of *E. histolytica*

The *E. histolytica* (strain HM1:IMSS) draft genome sequence was published and analyzed in 2005 (Loftus et al. 2005). The genome was later reassembled and reannotated, resulting in a genome assembly of 20,800,560 base pairs of DNA, distributed among 1496 scaffolds (Lorenzi et al. 2010; Clark et al. 2007). The genome has a high AT content, accounting for approximately 75% of its nucleotide composition, and is gene-rich, with approximately half of all assembled sequence being predicted to be coding sequence. A total of 8333 genes have been annotated.

C. Structure and organization of the genome

The genome assembly for *E. histolytica* comprises 20,800 base pairs of DNA distributed across 1496 scaffolds. This particular organism's genome is characterized by a high AT content, accounting for roughly 75% of its composition. Approximately half of the assembled sequences in the genome are predicted to encode proteins, with a total of 8333 annotated genes identified thus far. The structure of the *E. histolytica* genome has been extensively studied, with Clark et al. (Clark et al. 2007) providing a comprehensive review of its many interesting evolutionary features. Notably, the *E. histolytica* genome is highly repetitive, containing as many as 4500 copies of tandemly arrayed tRNA genes. The current genome assembly of *E. histolytica* is estimated to be 23.7 million base pairs in length, containing around 9,938 genes with an average size of 1.17 kilobases. These genes are distributed across 14 to 17 chromosomes, with variations in chromosome length attributed to the expansion and contraction of subtelomeric regions that may be formed by tandemly arranged tRNA sequences. The genome of *E. histolytica* is highly AT-rich, with an overall A+T content of approximately 75.3%. Furthermore, repeat sequences make up 6% of the genome in *E.*

histolytica. The genome of *E. histolytica* is characterized by the presence of both long interspersed repetitive elements (LINEs) and short interspersed repetitive elements (SINEs). While LINEs encode their own retro-transposition machinery, SINEs rely on the machinery provided by LINEs (Bakre et al. 2005). Most *E. histolytica* genes are comprised of a single exon, although up to 25% have the potential to be spliced, and 6% contain two or more introns. The short length of *E. histolytica* genes is primarily due to the loss of introns, and the 5' and 3' untranslated regions (UTRs) are typically brief. The average length of proteins predicted from the genome of *E. histolytica* is roughly 389 amino acids (Clark et al. 2007). Observations of the *E. histolytica* genome suggest that bacterial genes have been horizontally transferred into its genome and that some of these genes are involved in central metabolism. The occurrence of horizontal gene transfer from bacteria is not recent, as evidence suggests that it has been a part of the evolution of this remarkable parasite for some time (Roy et al. 2006). Furthermore, such transfers have likely played a significant role in shaping the genome of *E. histolytica* (Alsmark et al. in 2009). The genome of *E. histolytica* contains both linear and circular DNA molecules, as observed by various studies (Willhoeft and Tannich 1999; Riveron et al. 2000). One of these circular DNA molecules contains multiple copies of the rRNA gene in each nucleus (Bhattacharya et al. 1998). The *Entameba* genome is highly unstable in terms of chromosome ploidy, ranging from 4n-40n (Parfrey 2008). Additionally, the genome of this parasitic organism encodes a significant number of novel receptor kinases and features expansions of various gene families linked with virulence, such as cysteine and metallo-proteinases. These findings gleaned from the analysis of the *E. histolytica* genome offer new insights into the functioning and evolution of this major human pathogen.

D. Ribosomal RNA Genes

The nuclear ribosomal RNA (rRNA) genes in most organisms are arranged as tandem repeats on one or more chromosomes, and these repeats are highly repetitive. However, in *Entamoeba*, the rRNA genes are primarily located on extrachromosomal circular DNA molecules, and there is no clear indication of a chromosomal copy of these genes. This unusual location of rRNA genes in *Entamoeba* may be a direct result of cellular physiology, as studies with *Saccharomyces cerevisiae* mutants, where the rDNA is extrachromosomal, suggest (Bhattacharya et al. 1998). The structural RNA genes in *E. histolytica* are organized differently from those in most organisms. Specifically, the rRNA genes are exclusively carried on 24 kb circular episomes, which have two transcription units in an inverted repeat, as shown in **Fig**

2.4 (Bhattacharya et al. 1998). Typically, the expression of rRNA genes occurs in the nucleolus. However, previous studies have demonstrated that the rDNA circles are located at the nuclear periphery. It is believed that the circular episomes carrying the rRNA genes make up around 20% of the total cellular DNA in *E. histolytica*. Interestingly, approximately 15% of all the sequencing reads generated in the genome project were derived from this molecule, except for certain libraries where efforts were made to exclude it. While other circular DNA molecules of different sizes are suspected to exist in *E. histolytica*, with unknown functions (Dhar et al. 1995; Lioutas et al. 1995), they have not been identified in the genome shotgun sequence data as of yet.

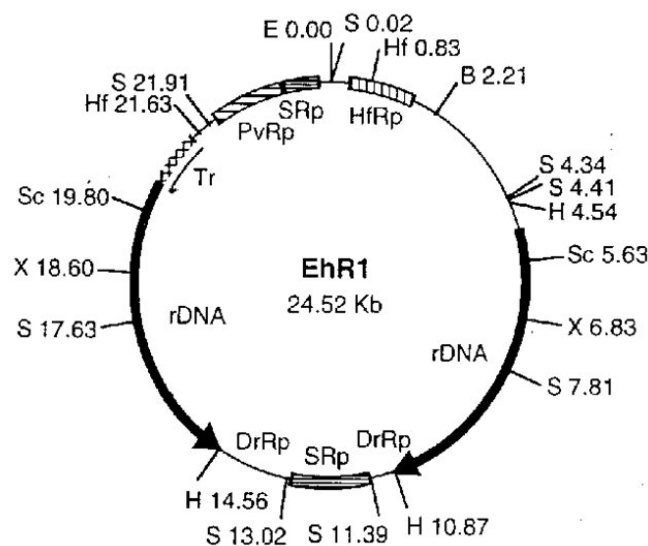


Fig 2.4 The figure illustrates the rDNA plasmid of *E. histolytica* HM-1:IMSS, highlighting the two rDNA inverted repeats and their transcriptional directions with bold arrows. The positions of nucleotides (in kilobases) are indicated at each restriction enzyme site, which includes BsaHI (B), EcoRI (E), HindIII (H), HinfI (Hf), ScaI (S), SacII (Sc), and XhoI (X). Additionally, the families of short, tandem repeats in the downstream and upstream spacers are abbreviated as DrRp for DraI repeats, SRp for ScaI repeats, PvRp for PvuI repeats, and HfRp for HinfI repeats. Overall, this figure provides a clear visualization of the essential features of the rDNA plasmid of *E. histolytica* HM-1:IMSS, facilitating a better understanding of the transcription and replication processes in this organism.

Source: Dhar et al. 1996; doi: 10.1128/MCB.16.5.2314.

E. tRNA genes

The genomic organization of *E. histolytica* is characterized by some distinctive features, which were discovered during initial investigations. The genome of this organism contains a high proportion of repetitive sequences, accounting for approximately 40% of the total genomic content. Notably, the number of tRNA genes in *E. histolytica* is exceptionally high, estimated to be around 4500 copies, which is roughly ten times greater than the number present in the

human genome. These tRNA genes are primarily organized into 25 distinct clusters, with most of the tRNA genes being arranged in tandem repeats. Each cluster comprises of repeated units that encode one to five tRNA acceptor types (Clark et al. 2006) The unit names of the tRNA arrays in *E. histolytica* correspond to the amino acid they encode, represented by the standard single-letter abbreviation and enclosed in square brackets. When necessary, the anticodon is also included to differentiate between different arrays. The intergenic regions within an array are identified using the same single-letter code and anticodon, but without the square brackets. For instance, in the *E. histolytica* array [ASD], both A-S and S^{GCT}-*D* are intergenic regions. The inclusion of the anticodon is essential to distinguish S^{TGA}-*D* from another array, [SD], which contains S^{TGA}-*D* instead. Furthermore, the STR loci in *E. histolytica* are named based on the tRNA genes flanking the intergenic region where they are located. For example, the STR locus S^{GCT}-*D* is situated in the intergenic region between tRNA genes for serine and cysteine. Additionally, the intergenic regions of these tRNA genes contain short tandemly repeated sequences (STRs) that resemble the micro/mini satellites present in eukaryotic genomes.

In contrast to randomly distributed micro/mini satellites, the STRs in *E. histolytica* are arranged in a tandemly arrayed larger unit (Tawari et al. 2008). Although clustering of tRNA genes has been observed in some other eukaryotes, the presence of tandem arrays of tRNA genes appears to be unique to *E. histolytica*. The origin of this unique gene organization remains a question of interest, and studies of tRNA gene organization in other *Entamoeba* species have been undertaken to address this question. Through genome surveys of four other *Entamoeba* species (*E. dispar, E. moshkovskii, E. terrapinae,* and *E. invadens*), researchers have gained a comprehensive understanding of their tRNA gene organization and have discovered a significant level of variation between these species.

E. histolytica/E. dispar comparisons

Entamoeba histolytica possesses two distinct versions of tRNA array that contain the genes for AsnGTT and LysCTT, which are referred to as (NK1) and (NK2). In contrast, the genome of its close relative, *E. dispar*, has only one type of (NK) array. Despite the identical tRNA/5S RNA gene content and orientation in all arrays of both species, the corresponding intergenic sequences display no significant similarity in their short tandem repeat (STR) or simple sequence regions. In contrast to the marked dissimilarity observed between the intergenic sequences of *E. histolytica* and *E. dispar*, *E. histolytica* displays significant variation within

its own species. Despite the noticeable differences in the number of short tandem repeats (STRs), the simple sequence regions and individual repeat sequences that constitute the STRs are remarkably conserved (Clark et al. 2007; Zaki et al. 2002; Ali et al. 2005)

***E. moshkovskii* Organization**

E. moshkovskii is closely related to *E. histolytica* and *E. dispar* (Silberman et al. 1999; Clark and Kaffashian et al. 2006) Therefore, it was unsurprising to observe that the majority of arrays in *E. moshkovskii* FIC possess the same gene content and orientation as those in *E. histolytica* and *E. dispar*. Specifically, 19 out of the 23 arrays in *E. moshkovskii* have the same arrangement as those in *E. histolytica* and *E. dispar*. Nonetheless, the array units in *E. moshkovskii* are considerably smaller on average than those in *E. histolytica* and *E. dispar*, with mean sizes of 64% and 61%, respectively.

F. Transposable elements (TES)

Transposable elements, also known as jumping elements, are segments of DNA that have the ability to move and insert themselves into new locations within chromosomes, as well as generate duplicate copies of themselves. They exist in both prokaryotic and eukaryotic cells and were first discovered in eukaryotes by Barbara McClintock in corn plants in 1940.These elements have significant biological importance as they can induce mutations in genes when they insert themselves into them, as well as influence gene regulation if they insert themselves near the promoter region. Moreover, they serve as a fundamental material for gene rearrangement, making them a crucial indicator in the evolution of the genome.

Similar to other organisms, *E. histolytica* possesses two types of transposable elements (TEs), namely long and short TEs. The long TEs are composed of three independent gene families that are arranged in tandem repeats, known as *E. histolytica* long interspersed nuclear elements (EhLINE). Meanwhile, the short TEs are composed of three gene families that are arranged in tandem repeats, but they are non-independent and are called *E. histolytica* short interspersed nuclear elements (EhSINE) (Al-Ardi 2021).

E. histolytica has two types of transposable elements, LINEs and SINEs, with sizes of approximately 4.8 kilobases and 0.5-0.7 kilobases, respectively. Together, they make up 11.2% of the organism's genome. The EhLINEs consist of a single open reading frame (ORF) with "CCHC" motifs, an endonuclease-like domain, and a reverse transcriptase (RT) domain.

Phylogenetic analysis of the RT domain places EhLINEs in the R4 clade of non-LTR elements, which includes members from nematodes, insects, and vertebrates. Interestingly, EhLINEs share a common 3' end sequence with EhLSINEs, suggesting that they may be involved in the retrotransposition of EhLSINEs.

There are three primary subtypes of autonomous LINEs in *E. histolytica*, namely EhLINE 1, 2, and 3. However, when examined, all copies of these EhLINEs encode non-conservative amino acid changes, frame shifts, and/or stop codons, with no continuous open reading frame (ORF) identified so far. This suggests that the majority of these elements are inactive or non-functional. Despite their inactivity, each EhLINE subtype is of nearly equal size, ranging from 4715 to 4811 base pairs (bp) in length, and can be interpreted as encoding a single predicted ORF that spans nearly the entire element. Specifically, EhLINE1 encodes 1589 amino acids, EhLINE2 encodes 1567 amino acids, and EhLINE3 encodes 1587 amino acids.It is worth noting that EhLINEs 1 and 2 are capable of mobilizing partner SINEs.

According to Bakre et al. 2005, there are two types of EhLSINE - EhLSINE1 and EhLSINE2 - which share a conserved 3' end sequence with EhLINEs. Additionally, EhLSINEs have a conserved 5' end that plays a role in their transcriptional regulation (Dellen et al. 2002). The length of EhLSINE1s varies, primarily due to differences in the number of internal 26-27 bp repeats. However, most EhLSINE1s contain two internal repeats and tend to be approximately 546 bp in length, as they are found clustered together. EhLSINE2, another type of SINE in *E. histolytica*, also has been reported (Van Dellen et al. 2002). In addition, a UEE1-like element has been identified in the *E. histolytica* genome and has been named EhLSINE3. Recent research by Yadav et al. suggests that *E. histolytica* has the ability to generate recombinant SINEs frequently during retrotransposition induced in vivo (Yadav et al. 2012).

Transposable elements such as SINEs can have an impact on the expression of nearby or neighboring genes through various mechanisms, including alternative promoter processing, splicing, and more. One distinguishing feature of SINEs is their stability across generations, and their insertion into genes is rare. Therefore, genetic analyses that utilize SINEs, such as those involving RFLPs and microsatellite sites, are considered fairly accurate (Al-Ardi 2021).

H. Large gene families:

E. histolytica has several large gene families that suggest their involvement in complex biological processes. One such family encodes a group of AIG1-like GTPases, which are of particular importance (Lorenzi et al. 2010). Although their exact functions are not yet fully understood, their differential expression suggests a possible association with virulence and/or adaptation to the intestinal environment (Gilchrist 2006; Davis et al. 2007; Biller et al. 2010). These GTPases are often located near transposons (Lorenzi et al. 2010). Another significant gene family encodes a large number (75-116) of leucine-rich repeat-containing proteins that are homologous to bacterial fibronectin (BspA of Bacteroides forsythus) and have a consensus sequence of LxxIxIxxVxxIgxxAFxxCxx (Davis et al. 2006b; Lorenzi et al. 2010).

Similar to another protozoan parasite *T. vaginalis, Entamoeba* encodes a significant number of Rab GTPases that are involved in vesicular trafficking within the cell. The large size of the gene family indicates the presence of complex vesicular trafficking machinery within the cell (Saito-Nakano et al. 2005; Nakada-Tsukui et al. 2010). The sequencing of the *E. histolytica* genome has identified more than 90 Rab GTPases. Phylogenetic analysis has revealed that 22 Rab proteins, including EhRab1-2, EhRab5, EhRab7-8, EhRab11, and EhRab21, exhibit significant similarities with Rabs from other organisms (Saito-Nakano et al. 2005).

The Gal/GalNAc lectin is an immunogenic surface antigen of *E. histolytica* that plays a crucial role in parasite colonization and cytotoxicity by binding with galactose and N-acetyl-D-galactosamine on the host cell surface. It is composed of a heavy chain subunit (hgl), a light chain subunit (lgl), and an intermediate chain subunit (igl) (López-Vancell et al. 2010). However, igl has not been detected in other species except for *E. histolytica* and *E. dispar* (Clark et al. 2007). The cysteine protease gene family is also present in both *E. histolytica* and *E. dispar* (Bruchhaus et al. 1996), but the key virulence determinant, cysteine protease 5, is a pseudogene in *E. dispar*. Southern blot analysis has revealed that the arie1 surface proteins of *E. histolytica* are either absent or highly divergent in *E. dispar*.

I. Gene number

The current genome assembly estimates that there are approximately 10,000 genes in *E. histolytica.* However, this number is subject to change with further improvements to the assembly and is expected to slightly decrease. Nonetheless, the relatively high gene count in *E. histolytica* compared to other parasitic organisms is indicative of its complexity and the

presence of large gene families, despite certain gene losses due to its parasitic lifestyle (Clark et al. 2007).

2.2.1.5. Central Dogma

A. Replication:

DNA duplication and chromosome segregation

E. histolytica cells have the ability to duplicate their genome multiple times before undergoing cell division. In axenic cultures, around 5-20% of trophozoites are multi-nucleated, with the exact percentage depending on the growth phase (Gangopadhyay et al. 1997). Moreover, DNA reduplication can occur in *E. histolytica* without nuclear division, leading to single nuclei containing 1X-6X or more genome contents (Das and Lohia 2002). These observations suggest that eukaryotic cell cycle checkpoints may be either absent or modified in *E. histolytica*, resulting in heterogeneity in genome content among axenically cultured trophozoites.

Initiation of DNA replication and DNA duplication

An in-depth study of the genome of *E. histolytica* has revealed that several proteins essential for initiating DNA replication are missing. These proteins include ORC 2-6, Cdt1, geminin, Cdc7/Dbf4, and Mcm10. However, a single gene encoding a homolog of the Cdc6/Orc1p protein found in archaeal and human cells has been identified in *E. histolytica* (Capaldi and Berger 2004). This finding suggests that the mechanism of DNA replication initiation in *E. histolytica* is similar to that in archaeal cells, where a single Cdc6p/ORC1p protein substitutes for the hetero-hexameric ORC complex (Kelman and Kelman 2004). Out of the four known checkpoint genes that regulate DNA replication in *S. cerevisiae*, only Mec1 and Mrc1 have homologues in *E. histolytica*. Several proteins involved in G1-S transitions, such as Sic1 and Chk1, do not have homologues in *E. histolytica*. In addition, the S-phase checkpoint genes p21, p27, p53, and retinoblastoma, which are necessary for the transition from G1 to S-phase in humans, are absent in *E. histolytica*, as reported by (Clark et al 2007). The Chk1 and Chk2 genes encode kinases that act downstream from the ATM and ATR kinases (intra-S-phase checkpoint genes). Although the Chk1 homologue is absent in E. *histolytica*, a Chk2 homologue has been identified and partially characterized in this organism (Iwashita et al. 2005).

B. Transcription

The genome of *E. histolytica* encodes 8201 protein-coding genes that include virulence genes and transcription factors associated with the basal transcription machinery. The promoters of several genes, such as hgl5, fdx, Ehpgp1, Ehpgp5, and actin, have been characterized, revealing important promoter elements like the TATA-box (GTATTTAAAG/C), Inr (AAAAATTCA), GAAC (AATGAACT), and GAAC-like (GAACTACAAA) core promoter elements, as well as other motifs like the H2O2-regulatory motif (HRM), URE-1, URE-3, URE-4, CCAAT-box, and CCCCCC motif. The GAAC-box is an unusual core promoter element that is crucial for initiating transcription at the promoter of the hgl5 gene, which lacks both TATA and Inr-boxes. Among the 246 genes analyzed, 43 contained the GAAC-box, 29 had the TATA-box, and 7 had both elements. Additionally, an in-silico analysis indicated that 56% of the 4000 genes analyzed possess a GAAC-like box, indicating that genome of *E. histolytica* is equipped with a diverse range of promoter elements that coordinate transcriptional regulation. Various transcription factors have been identified and characterized in this parasite, including EhTBP, EhEBP1, EhEBP2, EhHRM-BP, EhURE-BP, EhMyb, STAT, GATA, HSF, and the p53-like protein. Recently, EhPC4 was discovered to be a crucial player in regulating ploidy and genome stability in *E. histolytica*. Specialized functions in multicellular organisms have led to the evolution of TBP-related factors (TRFs) proteins that are related to the TATA-box binding protein (TBP). In *E. histolytica*, there are two tbp genes, Ehtbp1 and Ehtbp2, which produce proteins consisting of 234 and 212 amino acids, respectively. Although EhTBP2 is identical to amino acids 23-234 of EhTBP1, it is naturally silenced (Narayanasamy et al. 2018).

E. histolytica has an a-amanitin-resistant RNA polymerase II transcription, as reported by Lioutas and Tannich in 1995. Unlike other eukaryotic RNA polymerase II large subunits, the heptapeptide repeat (TSPTSPS) is absent from the C-terminal domain (CTD) of the *E. histolytica* protein. Additionally, the *E. histolytica* CTD is dissimilar to any other RNA polymerase II domain currently present in the database. *E. histolytica* mRNA molecules possess short 50 untranslated regions and have been found to be capped (Ramos et al. 1997; Vanacova et al. 2003). The discovery of homologues of Ceg1 RNA guanylyltransferase and Abd1 in *E. histolytica* provides new insight into the possible cap structure of newly transcribed RNA. Ceg1 adds an unmethylated GpppRNA cap while Abd1 methylates the cap to form m7GpppRNA (Clark et al. 2007). It has been suggested that these capping enzymes interact with the phosphorylated C-terminal domain (CTD) of RNA polymerase. Additionally, mRNA molecules in *E. histolytica* are polyadenylated, with the polyadenylation signal located in the short 30 untranslated region (Bruchhaus et al. 1993; Li et al. 2001).

C. Translation

There are two predicted tRNAs, IleTAT and Tyr in *E. histolytica.*, that contain introns and therefore require splicing. Unlike introns found in protein-coding genes, tRNA introns have a unique structure and require distinct splicing machinery. Fortunately, the necessary enzymes required for this splicing, known as tRNA modification enzymes, are present. Additionally, the tRNA synthetases required for aminoacylating the tRNAs are also present in *E. histolytica* (Clark et al. 2007). Most of the ribosomal protein genes in *E. histolytica* are highly conserved, with the exception of the gene for the large subunit protein L41, which could not be identified. In eukaryotic translation, the elongation factor (EF)-1 is activated when it binds to GTP, and it subsequently forms a ternary complex with aminoacyl tRNAs and ribosomes. The b and d subunits of EF-1 work as GDP-GTP exchange factors to facilitate the cycling of EF-1a between its two forms. Meanwhile, the EF-1g subunit provides structural support for the formation of this multimeric complex. EF2 plays a crucial role in the translocation of tRNAs on the mRNA by precisely one codon. While *E. histolytica* has most of the anticipated factors, it lacks EF-1d, a protein responsible for exchanging GDP with GTP. However, it is believed that EF-1b may perform this function instead. The EF-1 complex can exist in two forms: EF-1-a/b/g and EF-1-a/d/g, although in *E. histolytica*, it is likely that only the former complex is present. In eukaryotes, two polypeptide release factors, eRF1 and eRF3, are typically present. Both of these factors have been identified in *E. histolytica* (Clark et al. 2007).

2.2.1.6. Cell Cycle

A. Chromosome segregation and cell division

Yeast requires several proteins to form kinetochores, but these proteins have no clear counterparts in *E. histolytica*. This suggests that amoeba kinetochores may differ in composition and structure from those in yeast. The anaphase-promoting complex (APC) is responsible for regulating the transition from metaphase to anaphase. However, only APC11 could be identified in *E. histolytica*, as none of the other APC proteins were found in the organism (Clark et al. 2007). The APC complex is responsible for regulating cell cycle progression by targeting specific proteins for degradation via the proteasome pathway. However, in *E. histolytica*, most of the APC subunit homologues are absent. The study also found the presence of ubiquitin and related proteins, suggesting that the proteasomal degradation pathway for regulating cell cycle proteins may still be functional in *E. histolytica*.

During chromosome segregation, various novel microtubular assemblies, including multi-polar spindles, are involved. In *E. histolytica*, cytokinesis is accomplished by severing a thin cytoplasmic bridge, either independently or with the assistance of neighboring cells. Upon further growth, the separation of euploid and polyploid cells revealed that each sub-population acquired heterogeneous DNA content (Mukherjee et al. 2009).

B. CDKs and cyclins

The regulation of the cell cycle in different developmental stages and specific tissues is achieved through the association of various CDKs with specific cyclins. In the genome of *E. histolytica*, there are at least nine different CDKs present, but none of them contains the conserved PSTAIRE motif (Clark et al. 2007). Although eleven putative cyclin homologs with a high degree of divergence have been discovered in *E. histolytica* (Clark et al. 2007), their roles in the cell cycle and their corresponding CDK/cyclin partners remain unknown.

2.2.1.7. Proteome

Leitsch and colleagues conducted an initial exploration of the proteome of *E. histolytica* (specifically, the HM-1:IMSS virulent strain) using traditional two-dimensional (2D) SDS-PAGE and silver staining methods. Their work identified numerous proteins involved in various cellular processes and established the groundwork for future proteomic investigations of parasites (Bolaños et al. 2014*)*. A study visualized over 1500 protein spots on gels and identified several proteins through trypsin cleavage, followed by matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry and protein sequencing using Edman degradation. These identified proteins were found to be involved in metabolism, including pyruvate phosphate dikinase, fructose-1,6-bisphosphate aldolase, NADP-dependent alcohol dehydrogenase-1, and phosphoglycerate kinase. As anticipated, actin was observed to be one of the most prevalent protein spots on the SDS-PAGE gels. Trophozoites were found to contain a substantial quantity of superoxide dismutase, which is the primary detoxifying enzyme in *Entamoeba* (Marchat 2020).

MacCoy and Mann conducted a study in which they used trophozoites to identify proteins associated with Gal/GalNAc lectin. They bound the trophozoites to GalNAc-BSA-labeled magnetic beads and lysed them, then eluted the bound proteins for analysis using tandem mass spectrometry (López-Rosas et al. 2014). Their findings revealed the presence of several types of proteins, including cytoskeletal proteins such as actin, myosin heavy chain, and talin, as well

as signaling proteins like Rab11-B-related, calreticulin, and adenyl cyclase-associated protein. Additionally, they identified a novel transmembrane protein in their analysis. The initial investigations into amoeba proteomics showcased the high sensitivity of 2D gel electrophoresis and mass spectrometry, which facilitated the discovery of numerous noteworthy proteins. These studies laid the groundwork for future research in the field by establishing an experimental framework (Marchat 2020).

2.2.1.8. Pathogenesis

The name "histolytica" for this species is based on the Greek words for "tissue" and "dissolving," which aptly describes its ability to cause significant tissue damage. To initiate an infection, *E. histolytica* must first attach to the mucin layer of the colon. Trophozoites of the parasite produce a surface lectin that strongly binds to galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) oligosaccharides found on the host mucin and cells. Upon initial infection with *E. histolytica*, the mucosal layer may thicken as a potential defence mechanism to prevent direct contact between the parasite and the intestinal epithelium. *E. histolytica* is also capable of producing a mucin secretagogue, which stimulates the secretion of mucin from goblet cells, as well as glycosidases and proteases that can break down mucin polymers (Clark et al. 2007). When mucin is not present, the Gal/GalNAc lectin on the surface of the amoeba binds to exposed Gal and GalNAc residues on the intestinal epithelial cells (IECs). The progression of *E. histolytica* infection is characterized by a reduction in mucin levels, flattening of intestinal epithelial cells (IECs), and infiltration of neutrophils (Clark et al. 2007). Additionally, *E. histolytica* secretes molecules that can disrupt tight junctions and interfere with intestinal ion transport, leading to diarrhoea. Without the protective effect of mucin, *E. histolytica*-induced pathology is significantly more severe in murine models of amebiasis. In MUC2-deficient mice, the parasite is able to directly attach to IECs, causing even greater pathology, disruption of the intestinal barrier, and eliciting secretory and proinflammatory responses. As *E. histolytica* infection progresses, lesions in the intestinal epithelium can develop into flask-shaped ulcers that contain trophozoites, bacteria, and inflammatory cells. From these ulcers, trophozoites may invade the underlying lamina propria and enter the bloodstream, often leading to the formation of amoebic liver abscesses (ALAs) (Marie and Petri 2014).

A. Virulence Factors

To successfully invade and establish infection, *E. histolytica* must first cross the intestinal or hepatic barrier, then evade and overcome host immune responses, destroy host tissue, and migrate to subsequent microenvironments. Therefore, a successful infection requires the parasite to have a combination of defensive, offensive, and migratory capabilities. The events responsible for amoebiasis are not solely the result of the activity of a single virulence factor, but rather a coordinated action of various factors during the invasion process. Several important molecules have been studied for their role in tissue invasion, including adherence, cytotoxicity, cell killing, phagocytosis, and the onset of host immune responses.

Gal/Gal-Nac Lectin

The initial stage of contact-dependent lysis in *E. histolytica* involves the binding of a Gal/GalNAc inhibitable lectin to specific glycoproteins on host target cells. This lectin recognizes terminal galactose/N-acetyl-D-galactosamine residues that are exposed to the glycoproteins. The Gal/GalNAc inhibitable lectin is a complex consisting of three subunits, a heavy subunit (170 kDa), a light subunit (35/31 kDa), and an intermediate subunit (150 kDa) that is non-covalently associated with the other two subunits. The lectin has a molecular weight of 260 kDa (Mann 2002). The gene sequence of the heavy subunit contains a 15-amino acid hydrophobic signal sequence at the amino terminus. This is followed by a cysteine-rich extracellular domain consisting of 1209 amino acids that contain sites for N-linked glycosylation. The domain also shows significant similarity to the mammalian CD59 protein, which is involved in complement resistance. Furthermore, this domain includes an epidermal growth factor receptor-like tyrosine phosphorylation motif. The heavy subunit also has a transmembrane domain of 26 amino acids and a cytoplasmic domain of 41 amino acids (Mann et al. 1991; Tannich et al. 1991b). The heavy subunit is composed of approximately 6% carbohydrate. On the other hand, the light subunit is encoded by multiple genes that produce different isoforms with various posttranslational modifications. The two major isoforms are 35-kDa and 31-kDa, which have nearly identical amino acid compositions (McCoy et al. 1993). The 35-kDa light subunit isoform is highly glycosylated and lacks the GPI anchor found in the 31-kDa isoform. The light subunit gene family has 6-7 members, and their encoded proteins have a 13-amino acid signal sequence at the amino-terminus and a short 7-amino acid GPI-anchor addition motif at the carboxy-terminus. Adherence and killing of host cells in vitro were inhibited by genetic silencing of HGL, as well as the use of neutralizing antibodies and excess ligands (such as Gal and mucins). Furthermore, in humans, anti-CRD-IgA provided protection against reinfection (Marie and Petri 2014). In addition to the CRD, the HGL subunit also

contains an intracellular domain that shares homology with β-integrins, indicating a potential role in signalling following CRD engagement.

Amoebapore

Upon contact with mammalian cells in vitro, *E. histolytica* initiates a rapid cytolytic event that causes the inadvertent target cell to undergo swelling, surface blebbing, and lysis, while leaving the parasite unharmed. This extraordinary cytolytic activity is attributed to a 5-kDa polypeptide with pore-forming activity, which serves as the major effector molecule of *E. histolytica*. This molecule has also been implicated in apoptosis in target cells. (Leippe et al. 1991; Berninghausen & Leippe, 1997). The amoebapore is a polypeptide consisting of 77 amino acids with six cysteine residues. There are three types of amoebapore proteins, A, B, and C, present in a ratio of 35:10:1, respectively. Proteins A and B have a 57% amino acid identity, proteins A and C have a 47% identity, and proteins B and C have a 35% identity. (Bruhn & Leippe, 2001a). The most cytotoxic of the amoebapore proteins is Amoebapore C, which also displays strong antibacterial activity. Amoebapores are most effective at an acidic pH, with the optimal pH being 5.2. (Leippe et al. 1992). Amoebapores are stored in cytoplasmic vesicles and are only released upon contact with target cells (Leippe et al. 1995). They belong to a family of saposin-like proteins with conserved cysteine residues and lipid-interacting abilities (Bruhn & Leippe, 2001b). Inhibition of amoebapore A expression through antisense or gene silencing techniques reduces trophozoite cytopathic and cytolytic activities and impairs their bacteriolytic capability (Bracha et al. 1999). This suggests that amoebapore A is necessary for the full virulence of trophozoites and the formation of liver abscesses (Bracha et al. 2003)

KERP1

Upon penetrating the mucus layer, *E. histolytica* trophozoites come into contact with the intestinal epithelium, displaying a preference for the brush border of enterocytes. This indicates the possible existence of cellular components that serve as signals for tissue invasion (Seigneur et al. 2005). To identify new surface compounds involved in the pathogenesis of *E. histolytica*, researchers purified proteins that bind to the brush border. Among these, two previously unknown proteins enriched in lysine (K) and glutamic acid (E) were discovered and named KERP1 and KERP2, respectively. The further investigation focused on KERP1, which is unique and does not share homology with known proteins, including those of *E. dispar*. KERP1 is present in the trophozoite plasma membrane and internal vesicles and binds to host cell

surfaces. Studies on hamsters with experimentally induced ALA revealed that kerp1 gene expression is associated with hepatic amoebiasis. Using antisense RNA to inhibit kerp1 gene expression, researchers found that KERP1 abundance reduces under stress conditions, indicating complex gene regulation. The decrease in KERP1 abundance was found to correlate with a decrease in the modified ability of trophozoites to cause liver abscesses (Faust and Guillen 2012). Therefore, KERP1 is a critical factor in establishing amoeba-cell contacts and promoting liver abscess development, making it an essential component of the virulence of *E. histolytica*.

Cysteine proteases and the example of CP-A5

E. histolytica possesses a remarkable capability in its tissue invasion process, which involves the lysis of human cells and the destruction of the extracellular matrix (ECM). This ability is attributed to the presence of 80 genes in its genome that encode proteases, including 50 cysteine proteases (CPs) belonging to the papain superfamily (Tillack et al. 2007). The activation of CPs involves the removal of propeptide regions to facilitate proper enzyme folding, inactivation of the peptidase domain, and stabilization of the enzyme to prevent denaturation (Que and Reed 2000). CP-A1, CP-A2, CP-A5, and CP-A7 are the predominant and highly expressed proteases in axenic *E. histolytica* cultures, responsible for more than 90% of the proteolytic activity observed in trophozoite extracts (Irmer et al. 2009). These CPs play a direct role in tissue invasion by degrading both ECM proteins and mucin 2, the primary constituent of colon mucus. Moreover, they significantly contribute to immune evasion by breaking down host antibodies and complement components (Que and Reed 2000).

CP-A5, an exclusive cysteine protease of *E. histolytica*, plays a key role in the pathogenic process. It localizes at the amoebic surface (Jacobs et a. 1998), facilitates human colon invasion (Faust and Guillen 2012), and contributes to ALA formation (Faust and Guillen 2012). Notably, CP-A5 features an integrin binding motif, arginine glycine aspartic acid (RGD), in its propeptide region, which is also present in the proregion of cathepsin X in higher eukaryotes. RGD motifs in cell adhesion proteins like fibronectin act as recognition sites for cell surface receptors, including integrins. CP18 and CP112, although they possess the RGD integrin binding motif within their catalytic domains, are not abundantly expressed or secreted by *E. histolytica*. In experiments involving human colon explants exposed to *E. histolytica*, it has

been observed that CP-A5 is not essential for mucus degradation, although the presence of CP activity is necessary (Bansal et al. 2009). However, CP-A5 appears to play a role in subsequent stages of intestinal invasion that rely on the destruction of extracellular matrix (ECM) and the activation of human components induced or facilitated by CP-A5 (Thibeaux et al. 2012). The activity of CP-A5 is crucial for the conversion of pre-IL-1b into its active form. Subsequently, active IL-1b stimulates IFNg-sensitized cells to produce nitrogen compounds (Zhang et al. 2000). When the expression of the cp-A5 gene in amoeba was reduced through the use of antisense RNA or epigenetic silencing, their pathogenicity in the SCID-HU-INT model was diminished, establishing a connection between the pro-inflammatory activity of CP-A5 and intestinal invasion. The role of CPs in the formation of ALA was directly demonstrated by inhibiting their function using a specific inhibitor called trans-epoxysuccinyl-L-leucyl-amido-4-guanidino-butane (E-64), as well as by interfering with the expression of the cp-a5 gene (Ankri et al. 1999). To summarize, CPs, with a specific focus on CP-A5, play significant roles in pathogenicity. However, the interplay between their activity and that of other proteases, such as metallo- and serine-proteases, has not been thoroughly investigated yet.

Rhomboid proteases

Rhomboid proteases, which cleave transmembrane proteins within their transmembrane domains, have been associated with the process of host cell invasion (Urban, 2006). One specific rhomboid protease, known as *E. histolytica* rhomboid protease 1 (EhROM1), has been identified to possess distinct substrate specificity. EhROM1 primarily targets the heavy subunit of the Gal/GalNAc lectin found in amoebic cells. Interestingly, when the process of surface receptor capping is induced, EhROM1 relocates to the cap region (Baxt et al. 2008). Rhomboid proteases may have a new role in immune evasion by facilitating capping, a technique where parasite surface receptors recognized by host antibodies are polarized to the posterior end and subsequently shed.

Prostaglandin E2

A group of Canadian investigators conducted a study revealing that trophozoites of the parasite *E. histolytica* were capable of producing prostaglandin E2 (PGE2). This PGE2 synthesis, in turn, stimulated the release of interleukin-8 (IL-8) by colonic epithelial cells. Based on their findings, the researchers put forth a hypothesis suggesting that the elevated production of IL-

8, a powerful chemoattractant and activator of pro-inflammatory cells, played a pivotal role in initiating the development of amoebic colitis. In addition, the investigators demonstrated the involvement of prostaglandin E2 (PGE2) in the production of interleukin-8 (IL-8) by activating the prostaglandin E2 receptor 4 (EP4) in a manner independent of direct contact. Consequently, the PGE2 synthesized by *E. histolytica* was identified as a potential virulence factor contributing to amoebic colitis. Furthermore, the researchers proposed the use of EP4 inhibitors as novel therapeutic agents for the treatment of amoebic colitis (Abaza 2020).

Transmembrane kinases (TMKs)

Eukaryotic cells rely on transmembrane kinases (TMKs) for crucial cellular functions. TMKs consist of an extracellular transmembrane domain and an intracellular kinase domain, which can be either serine/threonine or tyrosine kinases. In the case of *E. histolytica*, TMKs are classified into six distinct families. The genomic analysis identified 35 members of the B1 family, which play a role in cellular proliferation. In an Indian study, researchers found that EhTMKB1-9 was located on the surface membrane of trophozoites. They also observed changes in trophozoite survival and growth when expressing EhTMKB1-9 without its kinase domain. Furthermore, the expression of EhTMKB1-9 was induced through a lipid-dependent signalling pathway, impacting endocytosis and cytotoxicity. These findings highlight the crucial role of EhTMKB1-9 (Abaza 2020).

Cytoskeleton and vesicle trafficking

The cytoskeleton of *E. histolytica* plays a critical role in its pathogenicity by regulating essential functions such as movement, tissue invasion, and host component phagocytosis. Filamins, actin-binding proteins involved in organizing filamentous actin networks, are particularly important in this regard. The overexpression of *E. histolytica* filamin (EhFLN) has been found to enhance motility and chemotactic response, indicating its involvement in cell motility and signaling processes (Diaz-Valencia et al. 2007). Kinesins, which are motor proteins, play a crucial role in the formation of spindles and the separation of genomes during cell division. In *E. histolytica*, silencing of EhKlp5, a unique member of the kinesin 5 family, through dsRNA interference, hampers spindle formation and cytokinesis (Dastidar et al. 2007). Vesicular trafficking regulated by Rab GTPases is also vital for parasite biology and pathogenesis. Rab GTPases have been implicated in the regulation of cysteine protease secretion and transport processes (Nakada-Tsukui et al. 2005; Mitra et al. 2007). Furthermore,

the overexpression of a mutated Rab protein in *E. histolytica* trophozoites led to a notable decrease in parasite phagocytosis, cytopathic activity, and the ability to generate liver abscesses in hamsters (Juárez-Hernández *et al.* 2013).

2.2.1.9. Host-Parasite interaction

The host-parasite relationship revolves around a complex interplay between the defence mechanisms of the host and the survival strategies of the parasite. While host cells employ various mechanisms to eliminate pathogens, amoebae have also evolved intricate strategies to manipulate the host's immune response and enhance their own chances of survival.

A. Cell-Mediated Immunity

Amoebic virulence is strongly influenced by the immune response, but surprisingly, *E. histolytica* can infect individuals with a fully functioning immune system. However, malnutrition, which weakens the immune system, appears to enhance the severity of the disease. On the other hand, in individuals with HIV/AIDS and impaired T cell immunity, the increased susceptibility to infection does not seem to result in more severe disease, which is still a puzzling observation. Additionally, it is unclear why men have a higher predisposition to developing amoebic liver abscesses (ALA) compared to women, despite both sexes being equally susceptible to the initial infection. The first line of defense against *E. histolytica* is the mucin barrier, which prevents trophozoite adherence and protects the intestinal epithelium from cytotoxicity (Chadee et al. 1987). When mucin is not present, trophozoites come into contact with the intestinal epithelium. The intestinal epithelial cells (IECs) detect the carbohydrate recognition domain (CRD) of the Gal/GalNAc lectin through Toll-like receptors 2 and 4 (TLR-2/4), which triggers the activation of NF-κB. This activation leads to the generation of inflammatory cytokines such as IL-8, IL-6, IL-12, IL-1β, IFN-γ, and TNF-α (Galva´n-Moroyoqui et al. 2011, Bansal et al. 2009). Amoebic lesions showed a higher presence of in vivo neutrophils compared to macrophages, as stated in reference. When activated in vitro, both neutrophils and macrophages exhibited amoebicidal activity. However, interestingly, *E. histolytica* demonstrated reciprocal killing. The resolution of infection was linked to the presence of IFN-γ, while IL-4 and TNF-α were found to be associated with the disease (Marie and Petri 2014). The correlation between the production of IFN-γ by peripheral mononuclear cells (PMNs) and protection against future *E. histolytica* disease in children was

found to be significant (Haque et al. 2007). In mice that were vaccinated, protection against the disease was observed when there were CD4+ T cells producing IFN-γ and CD8+ T cells producing IL-17 (Guo et al. 2009).

The prevalence of ALA in men may be influenced by testosterone-dependent mediation of IFN-γ. In experimental ALA, protection against the condition was facilitated by IFN-γ released by NKT cells, while macrophages producing TNF-α worsened tissue damage (Helk et al. 2013, Lotter et al. 2009). Female mice, with their higher levels of IFN-γ from NKT cells, exhibited greater protection against ALA (Lotter et al. 2013). However, both human and experimental studies have provided evidence suggesting that impaired cell-mediated immunity can exacerbate the damage caused by *E. histolytica* in various ways. In laboratory experiments, it has been observed that trophozoites can suppress cell-mediated immunity by eliminating immune cells and by enzymatically cleaving pro-IL-1β (resulting in activation) and IL-18 (resulting in degradation) (Marie and Petri 2014). Amoebic PGE2, when released into the surroundings, stimulates IL-8 production while reducing the expression of major histocompatibility complex II (MHC II) in macrophages. This process potentially hampers T-cell activation and diminishes the oxidative capacity of the cells (Wang et al. 1995).

B. Adaptive Immunity

The adaptive response of the immune system provides protection against *E. histolytica*, as demonstrated by reduced susceptibility to subsequent infections in mice following previous infection or vaccination (Guo et al. 2009). In humans, protection against reinfection is linked to the production of IgA antibodies (Haque et al. 2002). However, it is unclear why the presence of serum antibodies is associated with an increased frequency and severity of amoebic disease. *In vitro*, it was observed that antibodies could attach to *E. histolytica* and prevent its binding to host cells and molecules. Additionally, these antibodies, when bound to the surface of the parasite, triggered the activation of the complement membrane attack complex (MAC) (Marie and Petri 2014). However, *E. histolytica* managed to evade the immune defences mediated by antibodies by swiftly shedding the attached antibodies through a mechanism known as Gal/GalNAc lectin cap (Marie and Petri 2014). The Gal/GalNAc lectin hindered MAC formation on trophozoites and *E. histolytica* CPs broke down complement factors C3a and C5a, as well as host IgA and IgG.

C. Nutrition

Malnutrition leads to weakened immune systems and heightened vulnerability to *E. histolytica* infection. A notable correlation exists between malnourished or stunted children and the occurrence of *E. histolytica*-induced diarrhoea (Mondal et al. 2006). Moreover, malnourished children experience three times the number of *E. histolytica*-related diarrhoeal episodes compared to their well-nourished counterparts. Malnutrition specifically exhibits a significant association with *E. histolytica* infection, surpassing other types of gastrointestinal infections (Mondal et al. 2009). Mice that do not possess the leptin receptor (LEPR) in their intestinal epithelium exhibited comparable body weight, food intake, fecal microbiota composition, and expression of antimicrobial peptides. These findings suggest that the safeguarding effect is facilitated through distinct immune mechanisms regulated by leptin. These mechanisms are recognized for their involvement in preventing cell death, boosting mucin secretion, and promoting the mending and proliferation of intestinal cells (Marie and Petri 2014). Recent research suggests that *E. histolytica* infection could lead to nutritional deficiencies. This type of infection is linked to inflammation in the intestines, disruption of the mucosal lining, weakened barrier function, abnormal ion secretion, and an imbalance in the gut microbiota, all of which may have a cumulative impact on the nutritional well-being of the affected individual. Notably, infants who are breastfed have a reduced likelihood of contracting *E. histolytica* infections (Marie and Petri 2014). A recent study demonstrated that both human milk oligosaccharides and synthetic galacto-oligosaccharides provided protection to human intestinal epithelial cells against amoebic cytotoxicity in laboratory tests. Galacto-oligosaccharides, which are affordable and have good stability, are frequently included in infant formula. Consequently, these findings hold significant implications for nutritional interventions aimed at combating *E. histolytica* infections. (Jantscher-Krenn et al. 2012).

2.2.1.10. Clinical features

A. Symptomatic infection

Amoebiasis can manifest clinically either in the intestine or in extraintestinal organs, depending on the affected area.

B. **Intestinal infections**

There are four recognized clinical forms of invasive intestinal amoebiasis, all of which typically present as acute conditions. These forms include dysentery or bloody diarrhoea, fulminating colitis, amoebic appendicitis, and amoeboma of the colon.

Invasive intestinal amoebiasis is mostly characterized by dysenteric and diarrheic syndromes, which make up around 90% of the cases. Dysentery patients typically experience

moderate colic pain before passing three to five stools with a mixture of blood and mucus per day, accompanied by rectal tenesmus. On the other hand, patients with bloody diarrhoea have infrequent bowel movements, but their stools contain blood. Typically, fever and other systemic symptoms are not present in either case. Classic ambulatory dysentery syndromes can be easily distinguished from those caused by bacteria, as patients with bacterial dysentery often experience systemic signs and symptoms. These may include fever, chills, headache, malaise, loss of appetite, vomiting, cramping abdominal pain, and tenesmus (Stanley, 2003).

Amoebic colitis, which accounts for approximately 70% of cases, is commonly characterized by segmental ulceration in the colon. If perforation occurs, it primarily happens in the caecum (Stanley and Reed, 2001). As the ulcer progresses and deepens, it takes on the characteristic flask-like shape of an amoebic colitis ulcer, extending from the inner lining of the colon into the deeper layers of the tissue. Patients typically experience severe abdominal pain, persistent and intense urge to defecate (tenesmus), and over 20 episodes of bloody diarrhoea within a 24-hour period. Additional symptoms may include nausea, loss of appetite, fever, rapid heartbeat, and low blood pressure (Clark *et al.* 2007).

Amoebic appendicitis occurs in approximately 1% of adults living in regions where *E. histolytica* is widespread and is responsible for acute appendicitis cases. This condition arises when the parasite infiltrates the appendix located at the junction of the small and large intestines, leading to inflammation, tissue death, and potentially a rupture. Common symptoms include pain and tenderness in the lower right abdomen, fever, rapid heart rate, nausea, and vomiting. In some cases, involving the cecum, there may also be the presence of bloody diarrhoea. Amoeboma, a consequence of long-standing ulcers, is primarily found in the cecum, sigmoid colon, and rectum. Patients with amoeboma typically exhibit a painful and detectable abdominal mass along with bloody dysentery (Clark *et al.* 2007).

C. Extraintestinal

The most frequent extraintestinal manifestation of *E. histolytica* infection is an amoebic liver abscess. It can occur simultaneously with colitis, and approximately 10% of individuals with amoebic colitis are estimated to develop such abscesses (Stanley and Reed 2001). Common clinical symptoms include fever, chills, malaise, nausea, weakness, and a persistent, dull, aching pain in the right upper quadrant or epigastric region of the abdomen (Petri et al. 2000). Symptoms of amoebic liver abscess include right pleuritic or shoulder pain, weight loss, and myalgia. In rare cases, it can lead to obstructive jaundice if the biliary ductal system is

compressed. Hepatomegaly with palpation pain and point tenderness over the liver, ribs, or intercostal spaces are common clinical signs (Stanley and Reed, 2001).

In addition to the liver, the respiratory tract, brain, urinary, genital, and rectal tracts can be affected by amoebic infection. When the amoebic liver abscess ruptures through the diaphragm, it can cause pleuropulmonary amoebiasis. This complication is seen in approximately 7-20% of patients with amoebic liver abscess. Symptoms include coughing, pleuritic chest pain, and respiratory distress. In some cases, this condition can be misdiagnosed as bacterial pneumonia due to the presence of empyema and its effects on the lung tissue. Amoebic brain abscesses are extremely uncommon and almost always occur in conjunction with amoebic liver abscesses, accounting for less than 0.1% of liver abscess cases. The symptoms of amoebic brain abscesses include a sudden onset of symptoms such as headaches, vomiting, seizures, and changes in mental status. The progression of these symptoms can be rapid and, in severe cases, may lead to death (Ravdin, 1995).

D. Asymptomatic infection

Around 90% of individuals infected with *Entamoeba* experience no symptoms, making the infections largely asymptomatic (Kantor et al. 2018). The lack of symptoms of *Entamoeba* infections can be attributed to two main factors. First, it may be due to nonpathogenic strains of the parasite being responsible for these cases, as opposed to invasive infections caused by pathogenic strains. Alternatively, even in the presence of pathogenic strains, the course of infection can be influenced by host factors. Based on the available information, it seems that the level of key molecules rather than their complete absence in nonpathogenic species plays a more significant role in determining the property of pathogenesis.

2.2.1.11. Vaccine candidate

The absence of a suitable vaccine to prevent the transmission of the amebiasis parasite or the progression of infected individuals into invasive disease poses a significant challenge in controlling this disease. Currently, the development of vaccines for amebiasis is still in its early stages. However, several proteins from the amoeba have been identified as promising candidates for vaccination, as they have demonstrated the ability to effectively hinder or impede the formation of liver abscesses in rodents that were deliberately infected in laboratory experiments (Petri *et al* 1991; Zhang *et al* 1994; Soong 1995a; 1995b).

A. Gal-lectin

Gal-lectin, a 260-kDa heterodimer protein found on the surface of amoeba, has emerged as a highly researched antigen in the field of vaccine development. Its primary function is to act as an adhesin, facilitating the attachment of amoeba to cell surfaces. Numerous vaccine trials have been conducted using both native and recombinant forms of Gal-lectin, and these studies have demonstrated promising efficacy. In the initial experiment involving purified native Gal-lectin, an impressive 86% protection against amoebic liver abscess (ALA) was observed in the gerbil model. This finding highlights the potential of Gal-lectin as a valuable component in developing an effective vaccine against this disease. While Gal-lectin itself possesses strong immunogenic properties, it is crucial to employ appropriate adjuvants to enhance the immune response. Adjuvants play a critical role in stimulating a robust immune reaction, thereby improving the overall efficacy of the vaccine. Overall, the extensive research on Gal-lectin as a vaccine antigen underscores its significance and potential in combatting amoeba-related infections. Continued investigation and refinement of Gal-lectin-based vaccine formulations, alongside the exploration of novel adjuvants, hold promise for the development of more effective preventive measures against these diseases (Quach et al. 2014).

B. Serine-Rich *E. histolytica* Protein

In their study, Stanley et al. (1990) identified a protein called serine-rich *E. histolytica* protein (SREHP), which exhibited promising potential as a vaccine component. The researchers found that immunization with a fusion protein of SREHP and maltose binding protein (MBP), along with complete Freund's adjuvant, effectively prevented amoebic liver abscess when gerbils were challenged intrahepatically. Specifically, 64% of gerbils immunized intraperitoneally and 100% of gerbils immunized with a single subcutaneous injection were protected against the liver abscess (Zhang et al. 1994). Moreover, DNA vaccination using SREHP also demonstrated significant levels of protection against amoebic liver abscess in animal models of the disease (Zhang & Stanley 1999).

C. Lipophosphoglycans

The plasma membrane of *E. histolytica* contains lipophosphoglycan-like glycoconjugates, which are not found in *E. dispar*. These unique glycoconjugates hold promise as potential vaccine candidates, as demonstrated by the protective effects of a monoclonal antibody targeting them in experimental animal models of invasive amoebiasis (Marinets *et al.* 1997; Zhang *et al.* 2002; Melzer *et al.* 2003).

D. The Peroxiredoxin

According to Choi et al. (2005), within *E. histolytica*, there exists a cysteine-rich protein weighing 29 kDa. This particular protein functions as a peroxiredoxin, capable of deactivating hydroxyperoxide within host tissues. Remarkably, this molecule is 50 times more prevalent in *E. histolytica* when compared to *E. dispar*. Its abundance suggests a crucial role in safeguarding the parasite during its transition from the anaerobic conditions of the large intestine to human tissue. In a study, intraperitoneal application of recombinant peroxiredoxin in gerbils resulted in a systemic IgG response and provided 54% partial protection against *E. histolytica* trophozoite infection in the liver (Soong et al. 1995a). However, the safety of this product has not undergone assessment in primates.

2.2.1.11. Treatment

The main treatment for symptomatic amebiasis involves hydration and the administration of metronidazole and/or tinidazole (**Table 2.2**). The recommended dosages for these medications are as follows:

- ➔ Metronidazole: Adults should take 500 mg orally every 6 to 8 hours for a period of 7 to 14 days.
- ➔ Tinidazole: Adults should take 2 g orally once daily for a duration of 3 days.

In addition to these medications, luminal agents such as paromomycin and diloxanide furoate are also used in the treatment of amebiasis. In cases of amoebic liver abscess, a combination of metronidazole and aspiration guided by CT imaging can be employed. In certain situations where there is severe gastrointestinal bleeding, toxic megacolon, perforated colon, or liver abscesses that cannot be effectively drained percutaneously, surgical intervention may be necessary (Zulfiqar et al. 2023).

Tetracycline, chlortetracycline, oxytetracycline, and erythromycin can act as indirect amoebicides, primarily within the intestinal lumen. However, they lack efficacy in hepatic tissues and therefore are not employed for treating hepatic abscesses.

Table 2.2. Drug therapy for the treatment of amebiasis (Haque et al. 2003).

Disease	Drug	Dosage (mg/day)		Side effects
		Adult	Pediatric	
Amebic liver abscess	Metronidazole followed by a luminal agent	750 3 times	35–50 mg/kg of body weight in 3 divided doses	Primarily gastrointestinal: anorexia, nausea, vomiting, diarrhea, abdominal discomfort, unpleasant metallic taste; disulfuram-like intolerance reaction with alcohol, etc.
		For 7–10 days		
	Tinidazole followed by a luminal agent	800 3 times	60 mg/kg (maximum 2 g)	Primarily gastrointestinal and disulfuram-like intolerance reaction as for metronidazole
		For 5 days		
	Paromomycin	25–35 mg/kg 3 divided doses For 7 days		Primarily gastrointestinal: diarrhea, gastrointestinal Upset
	Diloxanide furoate	500 3 times a day	20 mg/kg 3 divided doses	Primarily gastrointestinal: flatulence, nausea, vomiting, pruritus, urticaria
		For 10 days		
Amebic colitis	Metronidazole followed by a luminal agent (as for amebic liver abscess)	750 3 times a day	35–50 mg/kg in 3 divided	As for amebic liver abscess
		For 7–10 days		
Asymptomatic intestinal Colonization	Paromomycin	25–35 mg/kg 3 divided doses For 7 days		Primarily gastrointestinal: diarrhea, gastrointestinal upset
	Diloxanide furoate	500 3 times a day	20 mg/kg 3 divided doses	Primarily gastrointestinal: flatulence, nausea, vomiting, pruritus, urticaria
		For 10 days		

2.2.1.12. Laboratory diagnosis

A. Intestinal infection

Sample collection and preservation

Effective sample collection and preservation methods play a critical role in determining the success of a diagnostic procedure. When diagnosing *Entamoeba* species, it is crucial to employ distinct approaches for the two diagnostic stages: cyst and trophozoite. **Fig 2.5** demonstrates that cysts are commonly found in well-formed stools, whereas trophozoites are typically present in watery or dysenteric stools. To facilitate the diagnostic process for intestinal protozoa in Sweden, **Table 2.3** provides a comparative overview of the commonly utilized fixatives in parasitology [Lebbad M. (2010) Molecular diagnosis and characterization of two intestinal protozoa: *Entamoeba histolytica* & *Giardia intestinalis*. PhD thesis]. It is important to note that none of the proposed agents are ideal for all techniques. While a recently collected stool sample directly from the patient is rarely accessible, it is crucial for detecting motile trophozoites. However, faecal samples are typically sent to diagnostic laboratories by mail, making it necessary to utilize fixatives to preserve the trophozoites.

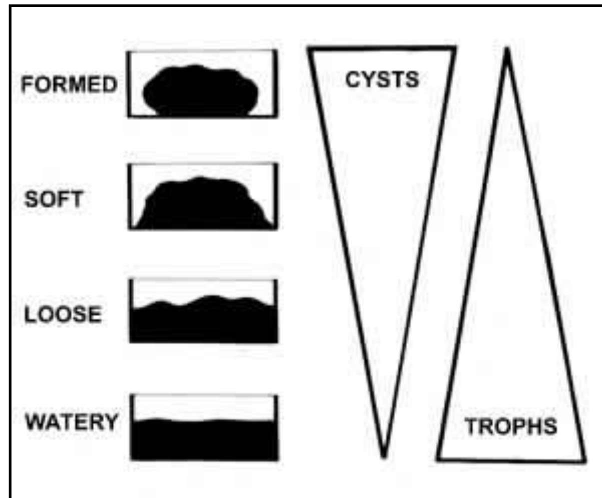


Figure 2.5 Distribution of cysts and trophozoites in relation to stool consistency.

(Source: http://www.dpd.cdc.gov/dpdx/html/DiagnosticProcedures.html)

Table 2.3 Comparison of different fixatives used to preserve stool parasites.

Analytical method	Unfixed sample	Formalin-fixed	SAF*-fixed	Ethanol-fixed
Direct microscopy of mobile trophozoites	Yes (fresh stool required)	No	No	No
Formalin/Ethyl acetate (ether) concentration	Yes	Yes	Yes	No
Permanent staining of trophozoites	Yes (if fixed immediately)	No	Yes	No
ELISA for *E. histolytica* antigen detection	Yes (less than 48 h)	No	No	No
Monoclonal antibody for *E. histolytica/E. dispar*	Yes	Yes	Yes	Yes
PCR	Yes (cysts)	No	No	Yes (cysts and trophozoites)

*Sodium acetate-acetic acid-formalin

Microscopy

For more than a century, microscopy has been the sole diagnostic method for identifying intestinal *Entamoeba* infection. Despite its inability to distinguish between *E. histolytica* and *E. dispar*, it remains the preferred technique in numerous parasitology laboratories globally. However, considering our current understanding, microscopy should be regarded as a

preliminary screening method for the *E. histolytica/E. dispar/E. moshkovskii* complex rather than a definitive means of confirming *E. histolytica* diagnosis.

Direct wet smear

Direct microscopy of a wet smear is a widely employed and cost-effective method for diagnosing intestinal protozoa worldwide. The necessary equipment consists of just a slide, a cover slip, and a drop of saline. Although reliant on the expertise of the microscopist, this technique is both rapid and affordable. However, it should be noted that a raw fecal sample contains numerous structures, including undigested food particles and human cells, which can potentially lead to misinterpretation as protozoan cysts or trophozoites. Nevertheless, when correctly interpreted, microscopy remains the simplest and most reliable technique for diagnosing invasive intestinal amoeba infections. Notably, the presence of *Entamoeba* trophozoites with ingested erythrocytes strongly suggests *E. histolytica*.

Concentration technique

The widely used technique for detecting faecal parasites is a variation of the formal/ether concentration method, initially developed by Ritchie in 1948 (Ritchie, 1948). Over the years, this method has undergone minimal modifications, primarily replacing ether with ethyl acetate (Young et al. 1979). Additionally, an alternative fixative called sodium acetate-acetic acid-formalin (SAF) can be employed instead of formalin (Yang and Scholten, 1977). Despite these minor adaptations, the fundamental principles of the technique have remained largely unchanged since its inception. The technique described concentrates amoeba cysts while trophozoites are only sporadically encountered.

Permanent staining techniques

Hematoxylin, trichrome, and chlorazol black dye are the most commonly employed staining techniques for identifying cysts and trophozoites in a lasting manner. In American literature, it is advised to incorporate permanent staining in all examinations for parasites and ova. On the other hand, European practices have historically focused more on cyst identification through concentration techniques. However, the resurgence of *Dientamoeba fragilis*, a protozoan associated with diarrhoea that lacks a cyst form, has prompted the adoption of permanent staining methods in Europe as well (Van Gool *et al.* 2003; Schuster and Jackson, 2009).

Molecular methods

Following the recognition of *E. dispar* as a distinct non-pathogenic species of *Entamoeba*, it has become evident that microscopy alone is unable to differentiate between *E. histolytica* and its non-pathogenic counterpart, *E. dispar*. Consequently, the utilization of molecular methods such as antigen tests and PCR has become crucial for accurate detection and differentiation (Ackers and Mirelman, 2006).

i. Antigen detection tests

Commercial antigen detection kits, such as *Entamoeba* CELISA PATH and TechLab *E. histolytica* II, employ monoclonal antibodies that target the Gal/GalNAc-specific lectin (adhesin molecule) of *E. histolytica*, enabling specific detection of the parasite. In Bangladesh, where *E. histolytica* transmission is prevalent, numerous studies have revealed that antigen detection exhibits a high sensitivity comparable to that of PCR (Haque *et al.* 1997; Haque *et al.* 1998). Conversely, in non-endemic regions, studies have indicated a lower sensitivity for antigen detection when compared to PCR (Gonin and Trudel, 2003; Stark *et al.* 2008). The reason behind the disparity in test results between areas where *E. histolytica* is common and areas where it is not remains poorly understood. One possible explanation is that PCR (Polymerase Chain Reaction) is significantly more sensitive in detecting trophozoites compared to antigen tests, with a detection sensitivity ranging from 100 to 1000 times higher (Mirelman et al. 1997; Stark et al. 2008). Based on current findings, it can be concluded that antigen testing, which offers rapid and technically simple results, is suitable for regions with a high prevalence of *E. histolytica* infection. However, it is not effective in settings where there are only a few cases of *E. histolytica* infection (Gonin and Trudel, 2003; Visser et al. 2006; Stark et al. 2008).

ii. Polymerase chain Reaction (PCR)

Initially, two distinct single PCR methods were employed to differentiate *E. histolytica* and *E. dispar*. One targeted the small subunit ribosomal RNA (18S rRNA) gene, while the other focused on the peroxiredoxin gene encoding a 30-kDa protein (Clark and Diamond 1991b; Tachibana et al. 1991). These methods were discussed extensively in an informative review written by Fotedar and colleagues in 2007 (Fotedar et al. 2007a). In recent years, numerous updated protocols have been developed for the detection and differentiation of *E. histolytica* and *E. dispar.* These include duplex PCR, multiplex PCR, nested PCR, and real-time PCR. Furthermore, various methods for detecting *E. moskhovskii* have also been described (Ali *et al.*

2003; Hamzah et al. 2006; Khairnar and Parija, 2007; Nazemalhosseini Mojarad et al. 2010). PCR assays for *Entamoeba* target various genetic loci, but the 18S rRNA gene has been the most commonly utilized for all *Entamoeba* species. This choice is primarily attributed to the multi-copy nature of the gene, which typically results in high analytical sensitivity.

B. Extra-intestinal amoebiasis

In cases of extraintestinal amoebiasis, it is uncommon to find *E. histolytica* parasites in stool samples (Fotedar et al. 2007a). Consequently, alternative diagnostic methods are necessary in such situations. Antibody detection is the preferred laboratory technique, and numerous serology methods have been developed for this purpose. Among these, enzyme-linked immunosorbent assay (ELISA) is the most widely employed. A recent study conducted in Bangladesh reported a sensitivity of 96% for patients with amoebic liver abscess (ALA) but only 46% for those with amoebic colitis, highlighting its varying performance in different clinical contexts (Haque *et al.* 2010).

Microscopy exhibits low sensitivity when aspirated abscess material is used for diagnosing *E. histolytica* infection. In contrast, both conventional and real-time PCR have demonstrated their value as reliable methods in such cases (Zaman *et al.* 2000; Khan *et al.* 2006; *Othman et al.* 2010). Invasive procedures ought to be minimized as they carry the potential for bacterial superinfection or unintended release of abscess contents. Therefore, their utilization should be restricted to situations where they are absolutely essential for confirming a diagnosis (Pritt and Clark, 2008). In a study conducted by Haque and colleagues in 2010, it was noted that the combination of real-time PCR analysis on non-invasive fluids such as urine and saliva achieved a sensitivity of 97% for ALA (amebic liver abscess) and 89% for amoebic colitis (Haque and colleagues 2010). Singh et al. proposed Loop-Mediated Isothermal Amplification (LAMP) as a fast and reliable diagnostic method for Amoebic Liver Abscess (ALA). LAMP offers high specificity, sensitivity, and simplicity, making it a promising approach for ALA diagnosis (Singh et al. 2013). This is particularly valuable in endemic regions where serology struggles to differentiate between past and current infections.

2.2.1.13. Epidemiology

Parasitic diseases have emerged as a major contributor to the burden of illness, resulting in significant health implications including mortality, affecting populations both in developing

and developed nations. These diseases are not confined to regions solely comprising high-income countries and have become a global concern (WHO, 2008).

Amoebiasis poses a significant threat to global health, particularly in tropical and sub-tropical countries with limited access to adequate healthcare services and sanitation infrastructure (Fotedar et al. 2012). The severe of disease complications, both intestinal and extra-intestinal, contribute to a substantial number of morbidity and mortality cases. Within a year, approximately 4 to 10% of individuals infected with the amoeba develop clinical symptoms, with amoebic dysentery ranking as the third leading cause of death among parasitic diseases worldwide, following Malaria and Schistosomiasis (Mortimer and Chadee, 2010; Ghasemi et al. 2015).

The prevalence figures of *E. histolytica* may be overestimated due to the inclusion of data that predates the distinction between the pathogenic *E. histolytica* and non-pathogenic species. It is possible that the presence of *E. dispar* and amphizoic *E. moshkovskii* could contribute to the reported prevalence, leading to a potential misinterpretation of the actual impact of invasive *E. histolytica* infection on mortality (Diamond and Clark 1993).

In 1986, Walsh reported that *E. histolytica*, a parasitic infection, affected approximately 10-20% of the world's population, with 1% of those infected developing the invasive form of the disease. The morbidity rate at that time was estimated to be around 100,000 people per year. Subsequently, according to reports from the World Health Organization (WHO), Pan American Health Organization (PAHO), and UNESCO in 1997, *E. histolytica* was found to affect approximately 500 million people globally, resulting in symptomatic diseases and causing the death of about 50 million individuals. However, it should be noted that 80-90% of the infections are asymptomatic, caused by non-pathogenic protozoa like *E. dispar* or *E. moshkovskii*. Therefore, the worldwide incidence of *E. histolytica* is more likely to be estimated at 5 million cases annually, with a global mortality rate remaining at 100,000 individuals per year (Jackson, 1998).

Entamoeba moshkovskii has gained significant attention in recent years due to its newfound ability to infect humans. Previously, it was widely regarded as a free-living amoeba without any significant medical implications. However, over the past decade, research has revealed its association with gastrointestinal symptoms (Shimokawa et al. 2012). This particular species of *Entamoeba* is commonly found in areas where amoebiasis is highly prevalent (Heredia et al.

2012). In the study conducted by Ali et al. 2003 in Bangladesh, a high prevalence of *E. moshkovskii* infections was identified. Among children aged 2 to 5 years, a prevalence of 21.1% for *E. moshkovskii* infection was observed, whereas 15.6% were found to have *E. histolytica* infection. Interestingly, it was noted that infection with *E. moshkovskii* is not uncommon and has a higher prevalence compared to *E. histolytica* infection. This trend was also observed in other regions, such as India (Parija and Kairnar, 2005), among homosexual patients with gastrointestinal symptoms in Australia (Fotedar et al. 2008), in two healthy adults in Tunisia, and in a cohort of HIV-suspected or confirmed patients in Tanzania (Beck et al. 2008). These studies further emphasized the significant prevalence of *E. moshkovskii* mono-infection.

Numerous instances of *E. histolytica* outbreaks have occurred globally due to sewage contamination. However, over the past three decades, the United States has not reported any outbreaks. From 1946 to 1980, six outbreaks related to waterborne and foodborne transmission were documented (Lippy and Waltrip, 1984). Notably, the most severe outbreak took place in Chicago in 1933, where defective plumbing resulted in sewage contamination of the drinking water system, leading to 1,000 cases, including 58 fatalities. Another significant incident involved the overflow of sewage water into the Swedish drinking water system during Christmas 1986, affecting over 1,400 individuals (Anderson and de Jong, 1989). In 1993, a private school in Taiwan witnessed an outbreak of amoebiasis, with 730 students affected due to contaminated underground well water (Chen et al. 2001). Tbilisi, located in the former Soviet Republic of Georgia, experienced a significant waterborne outbreak attributed to deficiencies in surface water filtration (Barwick et al. 2002). To address the situation, the city advised its population to boil drinking water, and several modifications were made to the water treatment process. These changes included: (i) enhancing the coagulation step, (ii) increasing chlorine doses, and (iii) reducing filtration velocities (United Nations Economic Commission for Europe, 2003). Despite these measures, the outbreak resulted in 106 residents developing hepatic abscess cases. In 1988, 42 Italian tourists visiting Thailand contracted infections caused by both *E. histolytica* and *Giardia* sp. The infections were attributed to the consumption of drinks containing ice, ice cream, and raw fruit served with ice.

The presence of *E. histolytica* and the types of amebiasis manifestations are widespread across the globe. In various regions, different forms of amebiasis predominate. For instance, in South Africa, the major form of amebiasis is the development of amoebic liver abscesses. In Egypt,

central and South America, Africa, and Asia, the intestinal invasive form of the disease is more common (Ravdin et al. 2003). In the Hue region of Vietnam, the incidence rates of amoebic liver abscesses were notably high, with approximately 21 cases per 100,000 inhabitants, coinciding with an *E. histolytica* infection rate of 11%. This higher incidence was linked to specific behavioural factors in the area, including increased alcohol consumption and significant passive food contamination by flies (Blessmann et al. 2002).

Amebiasis holds the fifth or sixth position among the top 20 causes of disease in Mexico on an annual basis. The reported incidence of amebiasis in Mexico varied from 1000 to 5000 cases per 100,000 inhabitants annually between 1995 and 2000, and from 615.85 to 1228.8 cases per 100,000 inhabitants between 2002 and 2006 (Ximenez et al. 2009).

In Egypt, a significant percentage of *E. histolytica* infections have been asymptomatic, exceeding 21%. In South Africa and Côte d'Ivoire, the rate of asymptomatic infection has ranged from 0% to 2% (Stauffer et al. 2006). Tunisia has reported asymptomatic infection rates between 0.85% and 1.86% (Chaker et al. 1995; Fathallah et al. 2004). In the northern part of Tunisia, approximately 2.15 cases per year were reported among individuals with diarrhoea (Bouratbine et al. 2003). These statistics reflect the conditions observed in other developing nations that face challenges such as inadequate sanitation infrastructure and insufficient health education. Consequently, resource-poor nations bear a significant burden of amebiasis and other diseases (Pritt and Clark, 2008).

Between 1990 and 2007, the death certificate data in the United States revealed 137 deaths caused by amebiasis, a disease characterized by higher mortality rates in males, older individuals, Hispanics, and Asians. California (36.27%) and Texas (22.16%), due to their large populations, significant immigrant populations, and proximity to endemic areas such as Mexico and Latin America, reported the highest number of deaths among the thirty states where amebiasis fatalities were identified (Gunther et al. 2011).

By conducting biochemical, immunological, and genetic analyses, researchers were able to accurately differentiate between *E. histolytica*, the pathogenic species, and non-pathogenic species. This separation provided valuable epidemiological data in areas where the disease is prevalent. The studies found that *E. dispar*, a non-pathogenic species, was up to 10 times more prevalent in asymptomatic patients compared to *E. histolytica* (Huston and Petri, 1999; Solaymani-Mohammadi et al. 2006).

In a refugee camp located in Dhaka, Bangladesh, a study conducted by Haque et al. 2006 revealed that among children aged 2 to 5 years, asymptomatic infection rates were 4.2% for *E. histolytica* and 13% for *E. dispar*. Similarly, in Aracaju, Brazil, Lawson et al. 2004 found that *E. histolytica* accounted for 1% of cases, while *E. dispar* was present in 13% of the cases analyzed. In Northeastern Brazil, Pinheiro et al. 2004 reported the absence of *E. histolytica*, but *E. dispar* was prevalent in 74.19% of cultured positive samples, as determined by PCR analysis. In a geographically isolated region of Ecuador, Gatti et al. (2002) conducted an isoenzyme analysis and found that *E. histolytica* accounted for 18.9% of cases, whereas *E. dispar* was present in 70.3% of cases. In northern Ghana, a microscopy-based study identified a high prevalence (39.8%) of *E. histolytica/E. dispar* complex, indicating the presence of both species (Haque et al. 2006). In India, the prevalence of *E. histolytica* has been reported to range from 0.5% to 38%. However, when molecular biology techniques were employed in the same subcontinent, the prevalence of this pathogenic protozoa was confirmed in 3.5% of the hospitalized individuals previously diagnosed with amebiasis through microscopy, out of a total of 11.7% (Khairnar et al. 2007).

Amebiasis represents a significant public health issue, particularly among children, in Saudi Arabia. In the city of Jeddah, during the period from December 1995 to October 1996, a study conducted by el Sheikh and el Assouli, 2001 found that the prevalence of *E. histolytica* was 2.2% among 576 fecal samples collected from children aged between 0 and 5 years who were experiencing acute diarrhoea. In the city of Makkah, located 70 km from Jeddah, a study by Al Harthi and Jamjoom, 2007 detected intestinal parasitic infections in 70.5% of cases between March and November 2005. The most common complex identified was the *E. histolytica/E. dispar* complex. Overall, these findings highlight the common occurrence of amebiasis and the prevalence of the *E. histolytica/E. dispar* complex in different regions of Saudi Arabia, with a particular focus on the high burden of the disease among children.

Between July 2010 and 2011, approximately 20% of the 1325 admitted cases were children suffering from *E. histolytica* gastroenteritis, according to Hegazi et al. in 2013. The confirmation of the disease primarily relied on the results of an antigen detection test, which demonstrated superior sensitivity and specificity compared to the traditional microscopic examination of stool samples. The authors attributed the observed increase in prevalence within the same locality of Jeddah, from 8.3% in 2007 to 20% in 2010, to the type of domestic water being used. It was found that a significant number of cases with higher infection rates

consumed water from wells designated for drinking or from home tanks utilized for other purposes. On the other hand, individuals who utilized desalinated water for these purposes were reported to have the lowest level of exposure to amebiasis (Omar et al. in 1995). An epidemiological study was conducted in Tunisia from 2002 to 2005 to determine the prevalence of *E. histolytica* in food handlers. A total of 4266 fresh stool samples were examined in the study. Microscopic examination revealed that 12 samples (2.8%) tested positive for the presence of *E. histolytica/E. dispar*. Subsequent DNA amplification of the SSU rDNA confirmed that 11 of these samples actually contained the non-pathogenic *E. dispar* (Ben Ayed in 2008b).

Among Human Immunodeficiency Virus (HIV) patients in several countries, the presence of *E. histolytica* was documented as follows: 13% in Colombia, 25.3% in Mexico, 10.3% in Ethiopia, 5.8% in Taiwan, and 12.4% in South Africa (Florez et al. 2003; Moran et al. 2005; Hung et al. 2008) (**Table 2.4**).

Table 2.4 Prevalence rate of *E. histolytica* reported from different parts of the world.

Entamoeba spp.	Prevalence	Technique	Reported from (year)
E. histolytica	1.5%	PCR	Iran (2011)[51]
E. histolytica	14.0%	PCR	Palestine (2011)[52]
E. histolytica	9.1% (Eh), 1.4% (Eh + Ed)	PCR	Malaysia (2012)[53]
E. histolytica	9.0%	PCR	Pakistan (2012)[54]
E. histolytica	10%	PCR	UAE (2013)[21]
E. histolytica	54.5%	Microscopy	Rwanda (2013)[55]
E. histolytica	10.2% (Eh), 3.3% (Eh + Ed)	PCR	Malaysia (2013)[56]
E. histolytica	36.6% and 19.4%	Antigen-based	Brazil (2013)[57]
E. histolytica	4%	PCR	Venezuela (2013)[58]
E. histolytica/dispar	48.0%	Microscopy	Saudi Arabia (2014)[59]
E. histolytica	22.55%	PCR	Cote d'Ivoire (2014)[60]
E. histolytica	0.55%	PCR	Colombia (2015)[61]
E. histolytica	39.4%	Microscopy	Egypt (2015)[62]
E. histolytica	28.2%	ELISA	Mexico (2015)[63]
E. histolytica	1.7%	PCR	Ethiopia (2017)[64]
E. histolytica	23.8%	PCR	Brazil (2016)[39]
E. histolytica/dispar	16.15%	Microscopy	Saudi Arabia (2016)[65]
E. histolytica/dispar	15%	Microscopy	Uganda (2016)[66]
E. histolytica	1.4%	Microscopy	Germany (2016)[67]

(Source: Nath et al. 2018; DOI: 10.18520/cs/v114/i10/2045-2052)

2.2.1.14. Amoebiasis in India

Limited research has been conducted in India regarding the prevalence of *E. histolytica*, *E. dispar*, and *E. moshkovskii* infections. Although sporadic studies have been performed, comprehensive analyses are scarce, especially following the re-description of *E. histolytica* and *E. dispar*, and the identification of *E. moshkovskii* colonization in humans. Detailed reports on the incidence of *E. histolytica* infection are available from only a few locations in India. The infection rates vary significantly across different regions, ranging from 0.5% to 12% (Rao et al. 1971; Joshi et al. 1980). Notably, *E. histolytica* infection appears to be consistently present throughout the year, with a peak observed during the summer and monsoon seasons (May to August) (Saha *et al.* 2007). Various studies conducted in different regions of India have reported microscopy-based prevalence rates of *E. histolytica*. For instance, Karnataka, Himachal Pradesh, and Tamil Nadu recorded prevalence rates of 14.8%, 42.0%, and 21.8% respectively. In Kolkata, Mukherjee et al. reported a prevalence rate of 3.6% among diarrhoeal patients using direct microscopy, PCR, and ELISA. Another study by Srivastava et al. in a New Delhi slum reported a prevalence rate of 8.8% among volunteers using PCR assay. Conversely, a comparatively lower prevalence rate of 1.7% was reported from Pondicherry using small sub-unit (SSU) rRNA gene-based PCR. A comparative study conducted in the same laboratory revealed a prevalence rate of approximately 19.9% for intestinal amoebiasis using microscopy. However, when nested multiplex PCR was employed to confirm the presence of *E. histolytica*, it was found in only 12.2% of the samples that tested positive. In an earlier study, we reported an overall prevalence rate of 13.7% among the population of Northeast India, using an integrated systematic molecular approach. In the current year, a seroprevalence rate of 15.38% was reported among suspected amebiasis groups in South India.

The majority of cases of Amoebic Liver Abscess (ALA) have been reported from the northern (Sharma et al. 2010; Sarda et al. 2011a; 2011b) and western (Singh et al. 2011) regions of India. Individual factors such as genetic background and dietary habits, including chronic alcoholism, may play a partial role in the occurrence of ALA. Chronic alcoholism can lead to increased hepatic iron deposition, and ALA cases are more commonly observed in livers with a high iron load. This has been suggested as a possible explanation for the higher incidence of ALA in alcoholic livers (Makkar et al. 2003).

A retrospective analysis of 86 hospitalized cases of Amoebic Liver Abscess presenting to the emergency department over a 5-year period was conducted (Sharma et al. 2010). The average age of ALA patients was 40.5 +/- 2.1 years, and there was a male-female ratio of 7:1. The mortality rate was 5.8%. However, cases of ALA recurrence (reported three times within a 5-year duration) have also been documented in northern India (Singal et al. 2012).

2.2.1.15. Molecular epidemiology and genetic diversity among parasite population

Extensive research has been conducted on the genetic variation among isolates of *E. histolytica*. This line of study is crucial as it may offer valuable insights into the reasons behind the majority of infections being asymptomatic, while only a few cases result in invasive diseases.

A. Isoenzyme analysis:

In earlier studies, isoenzyme electrophoresis was utilized to investigate the variation among *Entamoeba* species, specifically to differentiate between Pathogenic Zymodemes (now recognized as *E. histolytica*) and non-pathogenic ones (now recognized as *E. dispar*) (Sargeaunt and Williams, 1978; Sargeaunt et al. 1978). Among the 20 different zymodemes examined, only four zymodemes found in axenic cultures were deemed reliable for isolate typing (Jackson and Suparsad, 1997). However, isoenzyme analysis has become obsolete due to its time-consuming and laborious technique, as well as its reliance on established parasite cultures (Ali et al. 2008b).

B. Polymorphism based on repetitive markers:

Coding Loci

In the absence of microsatellite-like elements in the *E. histolytica* genome, genetic diversity and population structure studies have relied on alternative genetic markers such as the Serine Rich *E. histolytica* protein (SREHP) gene and Chitinase (Weedall and Hall N, 2011a). SREHP is an immune-dominant surface antigen involved in the phagocytosis of apoptotic host cells, thus preventing inflammatory responses by the host (Teixeira and Huston, 2008). Chitinase, on the other hand, is expressed exclusively during amoeba encystation (de la Vega et al. 1997a; 1997b). Both genes contain tandem repeats, which exhibit a high degree of inter-isolate diversity based on repeat types and arrangement patterns. Numerous genotyping studies have been conducted using these two markers (Ayeh-Kumi et al. 2001; Haghighi et al. 2002; 2003; Haghighi et al. 2008; Rivera et al. 2006). However, the SREHP locus demonstrates a

comparatively higher degree of polymorphism than the chitinase locus (Haghighi et al. 2003). The high genetic diversity within the SREHP locus, potentially attributed to immune evasion, may suggest a biological role (Stanley et al. 1991; Zhang et al. 1999; Zhang et al. 1994). It is important to note that PCR amplification of the SREHP locus can produce multiple and mixed PCR bands from a single strain due to allelic variation (Ali et al. 2008b), and the possibility of sequencing errors may contribute to the observed inter-isolate diversity.

Non-coding loci:

Polymorphism among axenic *E. histolytica* strains has been studied using RAPD (Rapid amplification of polymorphic DNA) analysis (Gomes et al. 2000). However, their utility as an epidemiological tool is limited due to inadequate resolution and the requirement for axenic culture of the parasite (Ali et al. 2008b).

E. histolytica possesses multiple copies of extra-chromosomal DNA circles encoding ribosomal RNAs (rRNAs) (Bhattacharya et al. 1988; 1989). In addition, a non-coding transcript known as the 'strain-specific gene' (SSG) is present upstream of the rRNA cistrons on these episomes (Burch et al. 1991). The SSG contains 26 bp tandem repeats that vary between *E. histolytica* strains (Stanley et al. 2001). However, some *E. histolytica* strains lack this episomal region, and *E. dispar* does not possess these genes, which limits their usefulness as genetic markers (Ali et al. 2008b).

C. tRNA linked short tandem repeat (STR) loci:

In contrast, tRNA-linked short tandem repeat (STR) loci have been widely preferred for evolutionary studies of *E. histolytica* due to their unique genetic organization and high susceptibility to recombination and mutation (Tawari et al. 2008). Previous studies proposed genotyping *E. histolytica* strains based on tRNA-linked STR loci, including D-A, STGA-D, N-K2, R-R, A-L, and S-Q (Ali, 2005; Zaki and Clark, 2001). However, these studies primarily relied on the variation in PCR product lengths of the target loci among *E. histolytica* isolates.

A more detailed sequence comparison of STR loci (R-R, STGA-D, and N-K2) among *E. histolytica* isolates was proposed by Tawari et al. 2008. Recombinational insertion or deletion within intergenic regions was suggested as a possible explanation for the observed differential band patterns. Each of the three studied loci contains blocks of repeats that exhibit both copy number and sequence variation. Moreover, within each block, there are often repeat sequence variants that differ by only one base. A total of 12, 17, and 18 repeat types have been identified

within the R-R (1RR-12RR), STGA-D (1SD-17SD), and N-K2 (1NK-18NK) loci, respectively. Detailed sequence analyses of the remaining three STR loci (D-A, A-L, and S-Q) among *E. histolytica* isolates have also been performed by Dr. C. Graham Clark at the London School of Hygiene and Tropical Medicine. A total of 15, 4, and 6 repeat types have been assigned within the D-A (1DA-15DA), A-L (1AL-4AL), and S-Q (1SQ-6SQ) loci, respectively.

Subsequent studies by different research groups from various parts of the world aimed to determine the parasite genotypes associated with disease outcomes. Previously reported repeat types were named according to their established nomenclature, and newly identified sequence types were assigned alphanumeric codes beginning with an alphabetical letter to indicate their geographic origin (Ali et al. 2007; Escueta-de Cadiz et al. 2010; Feng et al. 2012; Das et al. 2014; Jaiswal et al. 2014). A global genealogy of *E. histolytica* identified several haplotypes widely distributed across multiple countries (e.g., 5DA and 6DA found in Bangladesh, Japan, China, USA, India, etc.), while others were restricted to specific regions (e.g., 8DA and 11DA found only in Asian countries such as China, Japan, Bangladesh, etc.) (Zermeno et al. 2013). Similarly, certain haplotypes were found across all three forms of infection outcomes (e.g., 6DA associated with diarrhoea, asymptomatic infections, and liver abscesses), while others were specifically associated with certain diseases (e.g., J1DA from Japan found only in asymptomatic outcomes, VEN2DA from Venezuela associated exclusively with intestinal amoebiasis) (Zermeno et al. 2013).

D. Polymorphism based on SINE elements

The genome of *E. histolytica* contains a significant proportion of transposable elements, which can have a profound impact on the expression of nearby genes through various mechanisms. These elements can provide alternative promoters, splicing and polyadenylation sites, as well as induce heterochromatinization, thereby influencing the phenotypic outcomes of parasites (Kumari et al. 2011). Recent research by Kumari et al. 2013 has focused on studying the variability in the genomic distribution of SINE1 and SINE2 among clinical isolates of *E. histolytica*. They have identified multiple loci that exhibit extensive polymorphism in SINE occupancy among different strains of *E. histolytica*. Furthermore, they have proposed that analyzing the genomic locations of SINEs could serve as an advanced method for typing *E. histolytica* strains (Kumari et al. 2013).

E. Polymorphism based on non-repetitive markers:

Repetitive markers, although useful for regional and small-scale studies, are not efficient in probing the large-scale and long-term population structure of *E. histolytica* due to high rates of genetic diversity among parasite populations and frequent occurrence of novel genotypes (Weedall and Hall, 2011a). On the other hand, single nucleotide polymorphisms (SNPs) within non-repetitive loci are genetically stable and inherited by descendants, making them valuable for analyzing population structure. These SNPs show limited diversity, provide easily comparable and analyzable data (Beck et al. 2002; Weedall et al. 2012), and can be used to identify virulence factors associated with clinical outcomes (Bhattacharya et al. 2005; Gilchrist et al. 2012). SNP analyses can also help determine if a parasite population has experienced recombination events in its history (Weedall et al. 2012).

To investigate the genetic diversity of *E. histolytica*, various non-repetitive coding (*actin, tubulin, hgl3, amoebapore c, rabE* and *cysteine proteinase 5*), non-coding intron (*intron cta, intron cdc2c, intron 64.m00165* and *intron 128.m00017*), and intergenic regions were analyzed in isolates with different disease outcomes. The analysis revealed a higher number of SNPs in non-coding regions, possibly due to variable selection pressure. SNPs within coding regions were found to be significantly associated with clinical outcomes (Bhattacharya et al. 2005).

Comparative genomic hybridization studies between *E. histolytica* and *E. dispar* strains indicated relatively low genomic diversity within *E. histolytica*. A recent study by Weedall et al. also identified low levels of single nucleotide diversity within *E. histolytica* populations (Weedall et al. 2012). Similar observations were made through sequence analysis of defined regions (Bhattacharya et al. 2005; Ghosh et al. 2000). This low genetic diversity suggests a relatively recent common ancestor for *E. histolytica*. However, these findings contradict a recent report by Gilchrist et al. (Gilchrist et al. 2012). Multilocus sequence typing of *E. histolytica* clinical isolates revealed extensive population diversity, consistent with previous findings of high population diversity within repetitive regions. This suggests that the genotypes of individual parasites do not consistently show phylogenetic signals. The presence of genetic recombination events has been implicated in this phenomenon, as recombination can disrupt the linkage between target loci and lead to different genealogies (Gilchrist et al. 2012). Therefore, an important question regarding the population structure of *Entamoeba* is whether the parasite populations are predominantly clonal or undergo sexual reproduction.

2.2.1.16. Evidence for sexual reproduction:

While direct experimental evidence of genetic recombination in *E. histolytica* is lacking, several indirect pieces of evidence have been identified through population genetic studies. The complete genome sequencing of 10 axenic *E. histolytica* cell lines has revealed patterns of polymorphism that suggest past recombination events in the study population (Weedall et al. 2012). Additionally, bioinformatics comparisons of the Gal/GalNAc lectin gene between *E. histolytica* and its non-pathogenic relative, *E. dispar*, have provided evidence of gene conversion within lineages. Furthermore, the *E. histolytica* genome contains a set of genes necessary for meiosis, indicating the potential for sexual reproduction within natural populations (Loftus et al. 2005; Stanley, 2005). This feature is significant because sexual reproduction can facilitate the exchange of genes responsible for drug resistance and parasite virulence, potentially leading to the rapid spread of advantageous genotypes throughout the population (Weedall and Hall, 2011a).

Genetic recombination, involving the exchange of genetic material, has been reported in other protozoan parasites that were previously thought to be clonal, such as *G. lamblia* (Poxleitner et al. 2008; Cooper et al. 2007), *Leishmania major* (Akopyants et al. 2009), *Trypanosoma brucei* (Gibson et al. 2008), and *Trypanosoma cruzi* (Gaunt et al. 2003). Therefore, it is possible that *E. histolytica* also undergoes sexual reproduction. To further investigate this aspect, direct experimental evidence and more population genetic data from a single, putatively panmictic population would be valuable in addressing the question about the reproductive mechanisms of this intriguing parasite.

2.2.1.17. Association of parasite genotype with disease outcome

Population genetic studies of *E. histolytica* are primarily focused on exploring the genetic traits of the parasite that are associated with its virulence and differential disease-causing abilities. Genotyping studies using repetitive markers from various geographic regions have identified specific parasite genotypes linked to different disease outcomes (Ali et al. 2007; Escueta-de Cadiz et al. 2010; Feng et al. 2012; Das et al. 2014; Jaiswal et al. 2014). The initial evidence for a correlation between parasite genotype and outcome of *E. histolytica* infection was reported by Ali et al. 2007, although this study used PCR product size rather than DNA sequencing for genotype differentiation (Ali et al. 2007). Recently, Ali et al. 2012 reported a connection between sequence types of tRNA-linked R-R locus and disease outcome. Sequence type 5RR was mostly associated with asymptomatic outcomes, while 10RR was predominantly

associated with symptomatic outcomes such as diarrhoea and liver abscess. A genotyping study from Japan identified an avirulent *E. histolytica* strain with a unique tRNA-linked short tandem repeat (STR) pattern and suggested an association between *E. histolytica* virulence and tRNA-linked STR loci (Escueta-de Cadiz et al. 2010). The genotypes of *E. histolytica* in stool and liver abscess samples from the same patients were examined using tRNA-linked STR loci, revealing genetic distinctiveness between the two sample types. This suggests either varying organ tropism among parasite subpopulations within the same infection or DNA reorganization events within the parasite genome during tissue invasion (Ali et al. 2008a). However, contrasting and intriguing scenarios have also been reported, such as the genetic identity of *E. histolytica* strains isolated from amoebic liver abscess (ALA) patients and asymptomatic patients (Feng et al. 2012).

Nevertheless, it is important to note that tRNA-linked STR loci serve as surrogate markers for predicting infection outcomes (Ali et al. 2012). In contrast, outcome-specific single nucleotide polymorphisms (SNPs) identified within non-repetitive regions are more genetically stable and offer the possibility of directly identifying parasite virulence factors linked to disease outcomes (Gilchrist et al. 2012). Sequence analysis of defined regions has identified SNPs within the lectin (hgl3) gene that are significantly associated with disease outcomes. Lectin is considered an important virulence factor of *Entamoeba* (Bhattacharya et al. 2005). Whole genome sequencing of *E. histolytica* clinical isolates has also revealed two SNPs within the Cylicin 2 gene that are significantly associated with asymptomatic and liver abscess outcomes, highlighting Cylicin 2 as an important virulence determinant of *E. histolytica* (Gilchrist et al. 2012).

2.2.2 *E. dispar*

E. histolytica, a parasite, was first observed by Lösch in 1875 in Russia, as referenced by Jackson (1998). It received its formal name in 1903 by Schaudinn, as mentioned by Clark (1998). To explain the high prevalence of individuals infected with *E. histolytica* who showed no symptoms, Brumpt (1925), as cited by Walsh (1986), proposed the Dualist Theory. According to this theory, there were two amoebas that looked identical but were biologically different. It was originally thought that only one of them, *E. histolytica*, had the ability to invade tissues and cause amebiasis. However, further research using isoenzyme electrophoresis revealed that *E. histolytica* could be divided into two distinct groups: pathogenic and non-

pathogenic (NP). This classification was first described by Brumpt in 1925 and referenced by Sargeaunt et al. in 1978. The prevalence of asymptomatic disease in around 90% of infected individuals worldwide can be explained by the existence of the non-pathogenic (NP) species of *Entamoeba*. To establish the clear distinction between these two groups as separate species, DNA analysis was conducted. Diamond and Clark (1993) made reference to the earlier work of Brumpt (1925) when discussing this significant advancement. The newly identified species was named *E. dispar*. This differentiation was further supported by analyzing isoenzyme profiles, differences in gene sequences, and the application of monoclonal antibodies. The World Health Organization (WHO) officially acknowledged and recognized these findings as two distinct species.

Following the recognition of the species *E. dispar* in 1997, numerous studies were carried out to compare it with *E. histolytica*. Despite their morphological similarities, *E. dispar* has been identified as a separate species based on the analysis of isoenzyme patterns in amoebas obtained from asymptomatic individuals and those with invasive diseases. Both species of *Entamoeba* exhibit almost identical gene sets that encode major virulence factors. Nonetheless, a notable distinction arises in the secretion of cysteine proteases, where *E. histolytica* generates approximately 10 to 1,000 times more cysteine proteases compared to *E. dispar*. The amebaporesis activity of *E. dispar* is approximately one-third of that observed in *E. histolytica*, as stated in reference (Leippe 1997). Additionally, the Gal/GalNac lectin of *E. dispar* exhibits homology with two members of the heavy-chain family and four of the light-chain family, in contrast to the lectin of *E. histolytica*. This disparity results in reduced adhesion and cytotoxicity in vitro, as described in reference (Dodson et al 1997).

Before being identified as a distinct species, *E. dispar* was referred to as non-pathogenic (NP) *E. histolytica*, as mentioned in reference (Diamond and Clark, 1993). Experimental studies conducted on NP *E. histolytica* failed to demonstrate its ability to cause significant lesions in laboratory animals, as cited in references (Chadee et al. 1985; Vohra et al. 1989). Even following the recognition of *E. dispar* as an independent species, subsequent experimental investigations yielded comparable findings. In one study conducted by Espinosa-Cantellano et al. they conducted an experiment involving the inoculation of 5×10^5 and 5×10^6 *E. dispar* trophozoites into hamsters through the intra-hepatic route. After a seven-day period of infection, the researchers observed a mere focal inflammatory infiltrate without any signs of necrosis or granuloma formation (Espinosa-Cantellano et al. 1998). The discovery, combined

with laboratory observations, that *E. dispar* releases fewer and less potent toxic substances than *E. histolytica*, further supported the notion that *E. dispar* is a commensal organism incapable of causing harm to humans or inducing experimental lesions. In fact, when the *E. dispar* SAW 760 strain was cultured separately, it resulted in a less severe damage to MDCK epithelial cells compared to the damage caused by *E. histolytica*.

The exact prevalence of *E. dispar* remains uncertain. There is speculation that this species is responsible for a significant portion of infections that were previously attributed to *E. histolytica.* Research conducted globally has demonstrated a higher prevalence of *E. histolytica* in developing countries. However, even in these countries, there is a notable presence of *E. dispar*, indicating a high prevalence of this species as well (Evangelopoulos et al. 2001; Hegazi et al. 2013).

Amebiasis holds significant importance as a cause of morbidity in Brazil. In specific regions, such as Manaus, Fortaleza, and Belém, *E. histolytica* exhibits a higher prevalence. In Manaus, approximately 6.8% of the population is infected by this species (Benetton et al. 2005). In Fortaleza, the infection rate among the low-income population reaches 14.9% (Braga et al. 2001), while in the metropolitan area of Belém, it affects approximately 29.5% of individuals. However, in other parts of the country, studies indicate a higher prevalence of *E. dispar.*

In the regions of Pernambuco, Belo Horizonte (Minas Gerais), and Salvador (Bahia), the presence of only *E. dispar* has been detected, except for Minas Gerais. Our research team has identified an infection rate of approximately 5% with *E. histolytica/E. dispar* in communities located in the greater Belo Horizonte area (Gil et al. 2013). Thus far, 99% of these infections have been attributed to *E. dispar*. *E. dispar* is classified as non-invasive and is commonly associated with asymptomatic infections. In Brazil, non-dysenteric colitis is a prevalent clinical manifestation. The amoeba isolates from this particular group were examined using isoenzyme analysis and PCR, confirming their identification as *E. dispar* (Oliveira et al 2015).

Studies conducted in Brazil since 2000 using xenic *E. dispar* strains have revealed contrasting findings compared to earlier reports. In these studies, *E. dispar* strains obtained from patients in the North and Southeast regions of Brazil were injected into the livers of hamsters (at a dosage of 5 × 104 trophozoites) and into the ceca of rats (at a dosage of 1 × 106 trophozoites) (Gomes et al. 2000). Consequently, all the animals displayed hepatic and intestinal lesions

similar to those caused by *E. histolytica*, alongside the presence of trophozoites. Similar outcomes were observed by Costa et al. when they inoculated the livers of hamsters with 2.5 × 105 trophozoites of different *E. dispar* strains obtained from symptomatic and asymptomatic individuals in Brazil. Conversely, inoculating the microbiota of each strain into control hamsters did not induce liver lesions or resulted only in minor purulent lesions, suggesting that *E. dispar* trophozoites were responsible for the development of liquefactive necrosis. Importantly, the use of axenic strains and monoxenic strains of *E. dispar* failed to generate amebic abscesses, highlighting the significance of bacterial association in acquiring pathogenicity through mechanisms that remain unidentified (Clark et al. 2007).

The susceptibility of different hosts to *E. dispar* MCR strain was evaluated by introducing the strain into the liver and cecum. It was found that hamsters were the most susceptible, with all challenged animals developing an abscess caused by *E. dispar*. Both mice and rats exhibited amebic colitis in approximately 70% of cases (Oliveira et al. 2015).

The acquisition of pathogenicity in *E. dispar* strains may be attributed to their interaction with bacteria. It has been observed that the development of lesions occurs only when bacteria are associated with *E. dispar*, indicating that bacteria may induce initial injuries that promote the growth and invasion of trophozoites. This could be achieved by enhancing the expression of virulence factors and/or by transferring genes. Considering that *E. dispar* can cause significant experimental lesions in the presence of bacteria and the prevalence of intestinal co-infections affecting millions of individuals annually, it is crucial to conduct comprehensive studies to investigate the relationship between *E. dispar* and bacteria. These studies would shed light on the underlying mechanisms and provide valuable insights into this complex interaction. In the presence of bacteria, there was a notable increase in both the extent of intestinal necrosis and the intensity of the inflammatory response. When *S. typhimurium* alone was present, the inflammation in the sub-mucosal layer was considerably lower compared to animals co-infected with both *E. dispar* and *S. typhimurium*. Future molecular biology studies will be crucial in determining whether the bacteria facilitated the expression of amebic virulence factors and/or if the epithelial lesions caused solely by bacteria promote trophozoite adhesion and invasion. By conducting these studies, we can gain a deeper understanding of the intricate mechanisms underlying this phenomenon.

Further investigation is needed to fully understand the relationship between *E. dispar* and humans, as current evidence suggests. While *E. dispar* is generally considered a commensal organism in humans, there have been reports of its isolation from individuals experiencing non-dysenteric colitis symptoms (Martinez et al. 1996; Gomes et al. 1997) and even from a patient with dysenteric colitis (Graffeo et al. 2014). Additionally, DNA sequences belonging to *E. dispar* have been identified and genotyped in samples taken from patients with amebic liver abscesses, implying a potential role for *E. dispar* in the formation of lesions in the human intestine and liver. Consequently, more comprehensive studies are warranted to address this matter.

2.2.3 *E. moshkovskii*

Entamoeba moshkovskii belongs to the *Entamoeba* complex and shares a similar appearance to both *E. dispar* and the pathogenic *E. histolytica*. The World Health Organization (WHO) recommends treating only symptomatic (causing diarrhoea) and asymptomatic (without diarrhoea) forms of *E. histolytica* infection. To treat symptomatic *E. histolytica* infections, the recommended approach is to use metronidazole or tinidazole initially, followed by iodoquinol or paromomycin. As for asymptomatic infections, iodoquinol or paromomycin are typically used. Initially classified as a free-living amoeba, *E. moshkovskii* has been found to colonize humans in various regions over the years, including Yemen, India, Indonesia, Colombia, Malaysia, Tunisia, Tanzania, and Australia (Al-Areeqi et al, 2017; Parija and Khairnar 2005; Fonseca et. al 2016, Anuar et al. 2012; Ayed et. al 2008; Beck et al. 2008; Fotedar 2007). A study conducted by Beck et al. identified *E. moshkovskii* carriage in a population from Tanzania (Beck et al. 2008). In India, *E. moshkovskii* was reported to not cause diarrhoea but rather mild abdominal discomfort (Parija and Khairnar 2005). In Malaysia, *E. moshkovskii* was isolated from both symptomatic and asymptomatic individuals. A study conducted by Shimokawa et al. in 2012 (Shimokawa et al. 2012) suggested the potential pathogenicity of *E. moshkovskii* in causing diarrhoea in both mice and infants.

It has been found in various sources such as wastewater, freshwater rivers and lakes, brackish water, and human samples. When observed under a light microscope, its morphology cannot be differentiated from other *Entamoeba* species that parasitize humans, namely *E. histolytica* and *E. dispar*.

2.2.3.1. Structure:

The trophozoite form of this particular amoeba typically has a diameter ranging from 9 to 25 µm, but it is more commonly found to be between 11 and 13 µm. On the other hand, the cystic form of this amoeba has an average size that varies between 10.3 ± 0.1 and 11.82 ± 0.13, depending on the strain being studied. To examine its structure, researchers prefer to use iron hematoxylin staining. When stained, it is usually possible to observe 1-4 nuclei within the amoeba, with each nucleus measuring around 1.5-2 µm in diameter. However, there are also instances where the amoeba exhibits multiple nuclei. The morphology of the nuclei displays significant variability, including the presence of chromatoid bodies. These bodies can range in size from 3 µm to 7 µm per cyst, with an elongated shape and rounded edges. In mature cysts, these chromatoid bodies tend to occupy a central position (Neal, 1953).

2.2.3.2. Laboratory culture:

The growth conditions for *E. histolytica* involve a temperature range of 27°C to 36.5°C, whereas *E. moshkovskii* exhibits a broader temperature tolerance, ranging from 4°C to 40°C. In addition, *E. moshkovskii* possesses the ability to adapt to hypotonic cultures by developing a contractile vacuole, which is not typically observed under normal growth conditions (Goldman 1969; Neal 1953; Tshalaia 1941). For the laboratory culture of *E. moshkovskii* trophozoites, the BIS-33 medium is considered ideal. This medium supports the growth of *E. moshkovskii* under controlled conditions and is typically maintained at a pH of 6.8. For the cultivation of *E. moshkovskii*, both axenic and xenic culture media have been employed. Axenic culture media, such as TPS-1GM or TYI-S-33, are also utilized and supplemented with bovine serum at a concentration of 10%. On the other hand, xenic culture media like TYSGM-9, also supplemented with bovine serum but at a reduced concentration of 5%, or Robinson culture medium have also been employed.

2.2.3.4. History

In 1941, Tshalaia conducted the first characterization of *E. moshkovskii* using samples collected from various points within a wastewater treatment system in Moscow, Russia. During the study, Tshalaia isolated an amoeba strain that exhibited morphological similarities to *E. histolytica*, along with shared developmental features during excystation and the metacystic phase. However, notable differences between *E. moshkovskii* and *E. histolytica* were observed in their in vitro growth conditions. *E. moshkovskii* demonstrated the ability to grow within a temperature range of 4°C to 41°C, survive in hypotonic cultures, and thrive with limited

nutrient availability, conditions that were unsuitable for the growth of other *Entamoeba* species (Neal, 1953; Tshalaia, 1941).

Following the initial discovery of *E. moshkovskii* by Tshalaia, further investigations led to the identification of this amoeba in diverse regions worldwide (**Table 2.5**).

Table 2.5 Countries in which *E. moshkovskii* has been isolated.

Country	Sample type	Identification method	Year	Isolated by	Cited in
USSR	Wastewaters	Microscopy and culture	1941	L. Tshalaia	Tshalaia (1941)
USSR	Wastewaters, ponds, rivers	Microscopy and culture	1947	L. Tshalaia	Neal (1953)
USSR	Wastewaters	No information available	1947	V.G. Gnezdilov	Neal (1953)
Brazil	Wastewaters	Microscopy and culture	1949	A.D. Amaral R. Azzi-Leal	Ruiz (1960) Neal (1953)
England	Wastewaters	Microscopy and culture	1949	R.A. Neal	Neal (1953)
United States	Stool[a]	Microscopy and culture	1956	F.H. Connell	Dreyer (1961)
Canada	Wastewaters	Microscopy and culture	1959	P.J. Lachance	Lachance (1959)
Costa Rica	Wastewaters	Microscopy and culture	1960	A. Ruiz	Ruiz (1960)
Pakistan	Wastewaters	No data available	1967	V. Zaman	Scaglia et al. (1983)
Poland	Wastewaters	No data available	1960	Z. Hirschlerowa A. Swiecicki	Scaglia et al. (1983)
Malasia	Wastewaters	No data available	1962	V. Zaman	Scaglia et al. (1983)
United States	Stool[b]	Microscopy and culture	1963	M. Entner H. Most	Entner and Most (1965)
United States	Stool[c]	Microscopy and culture	1963	M. Entner H. Most	Entner and Most (1965)
Russia	Lakes	No data available	1958	L.I. Rogov	Felix-Silva (1971) Felix-Silva and Mayrink (1974)
Brazil	Wastewaters	No data available	1968	E. Félix-Silva	Felix-Silva (1971)
Brazil	River	Microscopy and culture	1971	E. Félix-Silva	Felix-Silva (1971)
Brazil	Fresh and wastewaters	Microscopy and culture	1964–1972	E. Félix-Silva W. Mayrink	Felix-Silva and Mayrink (1974)
Uruguay	Creeks	Microscopy and culture	1964–1972	E. Félix-Silva W. Mayrink	Felix-Silva and Mayrink (1974)
Australia	Wastewaters	No data available	1976	H. Scott B. McMillan	Scaglia et al. (1983)
Italia	Wastewaters	No data available	1963	I. De Carneri	Felix-Silva and Mayrink (1974) Scaglia et al. (1983)
Italia	Lakes and wastewaters	No data available	1966	I. De Carneri	Felix-Silva and Mayrink (1974) Scaglia et al. (1983)
Bangladesh	Stool	Riboprinting	1998	Haque et al.	Haque et al. (1998)
Bangladesh	Stool	PCR	2003	Ali et al.	Ali et al. (2003)
India	Stool	PCR	2005	Parija and Khairnar	Parija and Khairnar (2005)
Australia	Stool	PCR	2007	Fotedar et al.	Fotedar et al. (2008) Fotedar et al. (2007b) Stark et al. (2007)
Tunisia	Stool	PCR	2008	Ayed et al.	Ayed et al. (2008)
Tanzania	Stool	PCR	2008	Beck et al.	Beck et al. (2008)
Iran	Stool	PCR	2010	Nazemalhosseini Mojarad et al.	Nazemalhosseini Mojarad et al. (2010)
Colombia	Wastewaters	PCR	2011	Authors unpublished data	Authors unpublished data
Pakistan	Stool	PCR	2012	Yakoob et al.	Yakoob et al. (2012)

[a] Considered as *E. histolytica* Laredo strain.
[b] Considered as *E. histolytica* AG strain.
[c] Considered as *E. histolytica* JA strain.

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In 1947, Tshalaia successfully isolated an amoeba from a river in the city of Minsk, Belarus. This time, the isolation was not from a wastewater treatment system. The aim of Tshalaia was to distinguish it from *E. ranarum*, but attempts to infect tadpoles with strains of *E. moshkovskii* were unsuccessful (Neal, 1953). In 1950, Neal isolated *E. moshkovskii* from wastewater samples in London. Neal conducted a morphological description, established growth conditions, and tested the viability of the cysts. However, attempts to infect rats, guinea pigs, salamanders, and isolate the species from human stools were unsuccessful (Neal, 1953). In 1956, Pizzi in Chile conducted experiments using the same strain as Amaral and revealed that *E. moshkovskii* had the ability to phagocytose human, lamb, and rat erythrocytes. This discovery raised the possibility of a parasitic cycle for this amoeba (Pizzi, 1956). In 1961, Dreyer described an *Entamoeba* that had been isolated by Connel in 1956 from stool samples obtained from a patient in Laredo, Texas, who exhibited gastrointestinal symptoms. This marked the first documented case of human infection with *E. moshkovskii*. Dreyer characterized the amoeba as a strain of *E. histolytica* that exhibited unique characteristics. It could thrive in low-nutrient cultures at room temperature, displayed high osmotolerance, and showed resistance to emetine (Dreyer, 1961; Goldman, 1969). However, despite these distinct traits, it was not classified as *E. moshkovskii*. Instead, it was designated as the *E. histolytica* Laredo Strain due to its origin in Laredo, Texas. Subsequent human isolations with similar growth characteristics were grouped together under the label "*E. histolytica*-like."

Previously, *E. moshkovskii* was not regarded as a parasitic species since its isolations were primarily obtained from environments that indicated its status as a free-living amoeba (Neal, 1953; Tshalaia, 1941). However, certain researchers suggested that *E. moshkovskii* could be considered a parasitic species occasionally found in wastewater. They proposed that this amoeba, originating from human and animal waste, possessed the ability to survive and persist in wastewater due to its tolerance to varying tonicity and temperature conditions (Goldman, 1969).

An important study was conducted by Entner and Most in 1965, focusing on the characterization of two strains of *Entamoeba* isolated from humans. The first strain, named AG, was obtained from a patient suffering from chronic amebiasis that did not show a response to drug therapy. The second strain, named JA, was isolated from a patient in a psychiatric hospital. These two strains were compared to an *E. histolytica* strain (K-9 strain) and the *E.*

histolytica Laredo strain (*E. moshkovskii*). Entner and Most (1965) identified shared characteristics, including growth at room temperature and resistance to amebicides, among the AG, JA, and Laredo strains. They proposed that these strains be recognized as a new species, primarily due to their source of isolation and their classification as a parasitic species (Entner and Most, 1965).

In 1967, Stewart and Beck classified *E. moshkovskii* as a parasitic species due to the absence of the DNA-H antigen, which was found in all examined free-living species but absent in parasitic species (Stewart and Beck, 1967). While this discovery is significant, it is important to note that the absence of a specific feature alone is insufficient to ascribe a parasitic role to *E. moshkovskii*.

In 1969, Goldman suggested for the first time the possibility of *E. moshkovskii* infecting humans. This proposition arose when Goldman compared *E. moshkovskii* with both *E. histolytica* and *E. histolytica*-like species. He demonstrated that *E. moshkovskii* and *E. histolytica*-like shared similar biochemical, antigenic, and growth profiles, which rendered them virtually indistinguishable. However, based on these parameters, they exhibited significant differences from *E. histolytica*. As a result, Goldman proposed that infections caused by *E. histolytica*-like organisms should be referred to as "moshkovskii type *E. histolytica*" (Goldman, 1969).

In 1991, researchers Graham Clark and Louis Diamond utilized molecular analysis with the riboprinting technique to demonstrate that the strains previously identified in the literature as "*E. histolytica* Laredo" were, in fact, *E. moshkovskii* (Clark and Diamond, 1991). Subsequently, in 1998, Haque et al. also employed the same technique and reported a case of human infection with *E. moshkovskii*. In 1998, Haque et al. reported a case of *E. moshkovskii* infection in a five-year-old girl living in Bangladesh, a region recognized for its high incidence of intestinal diseases (Haque et al. 1998). This case served as the inaugural prevalence study conducted in the country, unveiling an infection prevalence rate of 21.1%. The results shed light on the widespread occurrence of *E. moshkovskii* infection in the area, underscoring its importance as a significant public health issue (Ali et al. 2003).

2.2.3.5. Diagnosis

The conventional approach for diagnosing *E. histolytica/E. dispar/E. moshkovskii* infection involves direct microscopic observation. However, this method has a significant drawback of low sensitivity, detecting less than 60% of cases, and it cannot differentiate between *E. moshkovskii* and the other two species. To overcome these limitations, alternative diagnostic methods such as culture and PCR-based techniques are recommended. The World Health Organization (WHO) specifically recommends the use of PCR-based techniques for accurate and reliable diagnosis (Fotedar et al. 2007a).

The culture-based diagnostic method for *E. moshkovskii* was established many decades ago and relies on its distinctive growth rate in culture, which differs from that of *E. histolytica* and *E. dispar* (Goldman, 1969). However, this technique has several limitations. Firstly, it is labour-intensive, requiring significant effort and resources. Secondly, its sensitivity is low, leading to a risk of missing positive cases. Thirdly, when mixed infections are encountered, it fails to discriminate between different species. Lastly, there is a high likelihood of cross-contamination with bacteria, fungi, and other protozoa, compromising the accuracy of the results.

Currently, the diagnostic approach for *E. moshkovskii* primarily relies on various PCR-based techniques. These methods specifically target the gene responsible for the small ribosomal RNA subunit (18S rDNA). This gene is a major focus due to its significant interspecies variability and the presence of multiple copies per genome, making it a reliable marker for distinguishing between different species (Fotedar et al. 2007a).

Riboprinting was the initial technique to utilize 18S rRNA for distinguishing *E. moshkovskii* from the other two species within the genus. However, one major drawback of riboprinting is its higher demand for labour and reagents compared to other PCR-based methods. This limitation hampers its suitability for analyzing a large number of samples.

Hamzah et al. reported a one-step PCR method that enables differential diagnosis of *E. moshkovskii*. This technique demonstrates the capability to detect DNA levels as low as 10 pg for both *E. moshkovskii* and *E. histolytica*, and 20 pg for *E. dispar*. Importantly, it exhibits no cross-reactivity with other parasites or bacteria that may induce similar gastrointestinal symptoms (Hamzah et al. 2006). The method described by Hamzah et al. may encounter

challenges in detecting mixed infections. This is because one primer is shared among all three PCRs in the multiplex. Consequently, if the quantity of DNA from one species is significantly lower, it may undergo limited amplification, leading to difficulty in visualizing the resulting product.

The Ali et al. method is widely employed for diagnosing *E. moshkovskii* and involves a two-step approach: nested PCR followed by restriction endonuclease digestion. This methodology offers several advantages, including enhanced test sensitivity and efficient amplification of stool DNA (Ali et al. 2003; Fotedar et al. 2007a). Notably, the method exhibits high sensitivity (86.6-100%) and specificity (100%) (Fotedar et al. 2007b; Khairnar et al. 2007). However, a potential drawback of this method is that a separate PCR is required for each individual species, making it a time-consuming procedure.

Khairnar and Parija introduced a nested multiplex PCR method that comprises two steps. In the first PCR, a genus-specific sequence is amplified, followed by a multiplex PCR in the second step, which allows for differentiation of *E. histolytica, E. dispar,* and *E. moshkovskii*. This method demonstrates variable sensitivity ranging from 38.7% to 94% and exhibits high specificity of 100% (Khairnar and Parija, 2007; Parija et al. 2010).

A recent development in the field is a multiplex real-time PCR method designed for the differential detection of *E. histolytica, E. dispar,* and *E. moshkovskii*. This approach involves the design of specific primers for each species, and differentiation is achieved simultaneously using a hybridization probe and melting curve analysis. One major advantage of this assay is that it eliminates the need for additional steps, thereby reducing the risk of amplicon contamination in the laboratory environment. Additionally, this method can detect very small amounts of DNA, with a sensitivity of 0.2 pg for *E. histolytica* and 2 pg each for *E. dispar* and *E. moshkovskii* (Hamzah et al. 2010). However, it is important to note that real-time PCR is generally considered a costly procedure.

The diagnosis of *E. moshkovskii* infections is crucial in present times, particularly in epidemiological settings, to assess its prevalence accurately and identify high-risk groups. This is essential to prevent the overestimation of the prevalence of the other two species. Moreover, these diagnostic methods can be employed to study the pathogenic role and other characteristics of *E. moshkovskii*, providing valuable insights into its behaviour and impact.

2.2.3.6. Epidemiology

An additional organism that closely resembles *E. histolytica*, called *E. moshkovskii*, has been documented to infect humans (as shown in **Table 2.6**). This finding holds significant importance as the primary diagnostic technique utilized for species identification is light microscopy, which has the potential to produce inaccurate results, leading to false positive diagnoses (Fotedar et al. 2007a).

In 2003, Ali et al. carried out the initial investigation that discovered *E. moshkovskii* in a particular population residing in Bangladesh. The study focused on 109 preschool children living in an area characterized by inadequate sanitation facilities and a high prevalence of amebiasis. Stool samples from these children were collected and subjected to various analytical techniques, including culture, isoenzyme analysis, antigen detection, and nested PCR. These methods were employed to identify and differentiate between various species of *Entamoeba* present in both single infections and co-infections. In this study, *E. moshkovskii* was found in 21.1% (23/109) of the samples, with 6/23 showing mono-infection. *E. histolytica* had a prevalence of 15.6% (17/109), and *E. dispar* had a prevalence of 35.8% (39/109). The results indicate that *E. moshkovskii* is not uncommon in this age group, as its prevalence surpasses that of *E. histolytica*. Additionally, *E. moshkovskii* is frequently detected as a coinfection with either *E. histolytica* or *E. dispar*. (Ali et al. 2003).

In 2005, Parija and Khairnar conducted an analysis in India, involving 746 samples from patients exhibiting clinical suspicion of gastrointestinal infection, which were initially examined by light microscopy. Among the samples where *E. histolytica/E. dispar/E. moshkovskii* were detected (68 samples), nested PCR was performed. The study reported an overall frequency of *E. moshkovskii* infection at 24.9% (17/68), with 1.4% (1/68) representing monoinfection cases. The frequency of *E. histolytica* was found to be 18.9% (13/68), while *E. dispar* had a frequency of 89.5%. These results align with the findings observed in preschool children in Bangladesh, indicating consistency across both populations (Parija and K hairnar, 2005). In their 2007 study, the same researchers analyzed 1720 samples from the general population. Among these samples, 202 tested positive for Entamoeba through light microscopy, culture, or both methods. An additional 35 negative controls were included, resulting in a total of 237 samples. PCR analysis was performed on the microscopy-positive samples and controls, revealing an infection rate of 15.6% (37/237) for *E. moshkovskii*, with 0.8% (2/237) representing mono-infections. *E. histolytica* was detected in 29.5% (70/237) of

the samples, while *E. dispar* was present in 72.2% (171/237) of the samples. The findings from this study differ from previous studies, with the frequency of *E. moshkovskii* being lower than that of *E. histolytica*. This difference could be attributed to the fact that the population under investigation exhibited gastrointestinal symptoms at the time of sample collection. However, these results are consistent with the observation that most *E. moshkovskii* infections tend to occur as co-infections with other species (Khairnar and Parija, 2007).

In a study conducted by Fotedar et al. in Sydney, Australia, in 2008, it was discovered that 50% of patients with gastrointestinal symptoms tested positive for *E. moshkovskii* infection using PCR. Out of a total of 110 patients, 55 individuals were identified as *E. moshkovskii* positive. Among the positive cases, 40% (22/55) were found to be monoinfected with *E. moshkovskii*, while 58% (32/55) exhibited mixed infections involving *E. moshkovskii*, *E. dispar*. Interestingly, only 2% (1/55) of the cases showed coinfection with both *E. histolytica* and *E. moshkovskii*. (Fotedar et al. 2007b, 2008). In Tanzania, Beck et al. conducted a study in the same year to investigate the prevalence of *E. histolytica, E. dispar,* and *E. moshkovskii* in stool samples from a group of inpatients suspected or confirmed to have HIV. The results revealed that 13.2% (18/136) of the individuals tested positive for *E. moshkovskii* using PCR, while 5.1% (7/136) tested positive for *E. dispar*. These findings highlight the importance of conducting further research on the HIV population to determine if there is a relationship between HIV and *E. moshkovskii* infections (Beck et al. 2008).

In 2010, Nazemalhosseini et al. conducted a study in Tehran, Gonbad, and Zahedan, cities located in Iran, to investigate the prevalence of *E. moshkovskii* infection. Among the 58 individuals tested, the infection frequency was found to be 5.2% (3/58). Out of these cases, two samples indicated mono-infection with *E. moshkovskii*. Similarly, a more recent study conducted in Pakistan in 2012 aimed to assess the prevalence of *E. histolytica, E. dispar*, and *E. moshkovskii* in individuals experiencing chronic diarrhoea accompanied by abdominal pain or discomfort resembling irritable bowel syndrome. Among the 318 individuals tested, PCR analysis revealed a positive result for *E. moshkovskii* in 13.2% (42/318) of cases, *E. histolytica* in 6.6% (21/318) of cases, and *E. dispar* in 18.6% (59/318) of cases. However, the study did not provide information regarding the rates of coinfection between these different species.

The available studies on *E. moshkovskii* indicate that this infection is not considered atypical but rather common, particularly among individuals who have risk factors for amebiasis. These risk factors include poor sanitary and socioeconomic conditions, extremes of age, and

malnutrition (Pritt and Clark, 2008; Stanley, 2003). It is worth noting that the frequency of *E. moshkovskii* infection varies significantly among different study groups, which can be partly attributed to differences in sanitary conditions, lifestyles, and the specific populations being studied (Ali et al. 2003; Beck et al. 2008; Fotedar et al. 2008; Parija et al. 2010; Yakoob et al. 2012). Therefore, conducting additional prevalence studies is crucial to accurately assess the true epidemiological impact of this infection.

Table: 2.6. Prevalence of *E. histolytica/E. dispar/E. moshkovskii* complex infection in humans.

Reference	Population	No. of individuals	Total prevalence[a]	Prevalence by species[b]		
				E. moshkovskii[c]	*E. histolytica*[c]	*E. dispar*[c]
Ali et al. (2003)	Children aged from 2 to 5	109	68.8% (75/109)	21.1% (23/109)	15.6% (17/109)	35.8% (39/109)
Parija and Khairnar (2005)	Patients with gastrointestinal symptoms	746	9.1% (68/746)	24.9% (17/68)	19.0% (13/68)	96.8% (66/68)
Khairnar and Parija (2007)	Patients with gastrointestinal symptoms	1720	11.7% (202/1720)	18.3% (37/202)	34.6% (70/202)	84.6% (171/202)
Beck et al. (2008)	Patients with a suspected or confirmed HIV infection	136	20.6% (28/136)	13.2% (18/136)	4.2% (5/118)[d]	5.1% (7/136)
Fotedar et al. (2008)	Patients with gastrointestinal symptoms	5921	1.9% (110/5921)	50.0% (55/110)	4.5% (5/110)	57.3% (63/110)
Nazemalhosseini Mojarad et al. (2010)	Patients with gastrointestinal symptoms	3825	1.5% (58/3825)	5.2% (3/58)	5.2% (3/58)	93.1% (54/58)
Parija et al. (2010)	Patients with gastrointestinal symptoms	246	19.9% (49/246)	8.2% (4/49)	12.2% (6/49)	28.6% (14/49)
Yakoob et al. (2012)	Patients with symptoms mimicking IBS and healthy controls	318	39.3% (125/318)	13.2% (42/318)	6.6% (21/318)	18.6% (59/318)

[a] Includes methods other than PCR.
[b] Determined by PCR.
[c] Includes coinfection.
[d] Determined by ELISA.

(*Source:* Heredia et al. 2012; *https://doi.org/10.1016/j.actatropica.2012.05.012)*

2.2.3.7. Clinical perspectives:

The discussion surrounding the pathogenicity of *E. moshkovskii* gained prominence in 2008 when Fotedar et al. conducted a study. In their research, they identified three patients who exhibited gastrointestinal symptoms such as diarrhoea and abdominal pain. These patients were found to be infected with *E. moshkovskii* through PCR analysis. Notably, tests conducted to detect commonly prevalent gastrointestinal pathogens and other species within the *Entamoeba* genus yielded negative results. (Fotedar et al. 2008). Two additional studies conducted by Parija and Khairnar, as well as Nazemalhosseini et al. 2010 reported cases of patients with *E. moshkovskii* monoinfection exhibiting symptoms similar to those described in the study by Fotedar et al. However, these studies had limited depth in terms of analyzing other pathogens that could be associated with these symptoms. The authors mentioned that bacterial analysis was conducted on the samples, but they did not provide specific details regarding the bacteria tested or the methods employed. Notably, viral tests were not performed in these studies (Nazemalhosseini Mojarad et al. 2010; Parija and Khairnar, 2005).

In their study, Yakoob et al. conducted an investigation on the prevalence of *E. histolytica/ E. dispar/ E. moshkovskii* in patients experiencing chronic diarrhoea along with abdominal pain or discomfort (Yakoob et al. 2012). They specifically focused on individuals who had already tested negative for bacterial and other parasitic infections. The study revealed a significant correlation between chronic diarrhoea and *E. moshkovskii* infection. Notably, the research emphasized the evaluation of non-infectious factors contributing to chronic diarrhoea in the population, such as celiac disease, thyroid dysfunction, and chronic pancreatitis. However, the study did not provide information regarding the prevalence of these conditions or whether patients affected by them were excluded from the statistical analyses. Moreover, the exact rates of coinfection remain uncertain, and it would be valuable to understand the importance of the connection between chronic diarrhoea and *E. moshkovskii* monoinfection (Yakoob et al. 2012). Consequently, it is difficult to determine the role of *E. moshkovskii* in the initiation of symptoms.

In a study conducted by Beaver et al. the experimental infection of *E. moshkovskii* was investigated. The researchers infected 42 healthy individuals, who were prisoners at a state penitentiary, with an average inoculum of 2000-4000 *E. moshkovskii* cysts. At that time, *E. moshkovskii* was believed to be a strain of *E. histolytica*. Following the infection, every participant experienced a prepatent period ranging from 7 to 14 days. The infection became patent between 21 days and 9 months. Interestingly, none of the patients exhibited any symptoms throughout this period, and the infection resolved spontaneously (Beaver et al. 1956b). These findings could potentially lend support to the hypothesis that *E. moshkovskii*, similar to *E. dispar*, is a non-pathogenic parasite. However, it is important to note that due to limitations at the time of the study, we cannot definitively confirm whether the cyst inoculum or the samples collected during follow-up were indeed *E. moshkovskii*. Additionally, the presence of *E. histolytica* or *E. dispar* in the initial inoculum could potentially have influenced the course of the infection.

The clinical implications of this phenomenon, particularly in the context of mixed *E. histolytica* infection or the pathogenicity of *E. moshkovskii*, remain unknown. However, considering previous cases, it is essential to evaluate how *E. moshkovskii* responds to current drugs. The study by Yakoob et al. provides the only evidence of successful treatment for *E. moshkovskii*, wherein patients were treated with metronidazole and diloxanide, leading to resolution of the

infections. Further assessment of the drug response of *E. moshkovskii* is warranted (Yakoob et al. 2012).

Hence, conducting further studies is crucial to precisely define the symptoms that need to be examined and to ensure strict exclusion criteria for patients with infectious and non-infectious diseases that may present similar clinical manifestations.

2.2.4 *E. bangladeshi*

In 2011-12, a significant research study conducted in Bangladesh led to the identification of a new species called *Entamoeba bangladeshi*. This species was named as an expression of gratitude towards the supportive Bangladesh community. The study involved collecting faecal samples from a cohort of children residing in Mirpur, encompassing both those with diarrhoea and those under surveillance(Royer et al 2012; Gilchrist et al 2014).

The study findings unveiled the existence of an entirely unique species within the *Entamoeba* genus. Analysis of the SSU rRNA gene revealed substantial dissimilarities between this novel species and all previously sequenced *Entamoeba* species. As a result, these isolates were classified as a distinct species of *Entamoeba* and given the name *E. bangladeshi*.

In terms of evolutionary relationships, *E. bangladeshi* exhibited greater genetic divergence compared to the non-invasive *E. dispar*, while being closer to *E. histolytica* than *E. moshkovskii.*

E. bangladeshi was successfully established in xenic culture, demonstrating its capability to thrive at both 37°C and 25°C. This characteristic is shared with *E. moshkovskii* and *Entamoeba ecuadoriensis*, setting it apart from *E. histolytica* and *E. dispar*. Under light microscopy, there are no observable distinctions between *E. bangladeshi* and *E. histolytica*. However, it can be distinguished from other known Entamoeba species through the application of immunologic and molecular techniques (Royer et al 2012; Gilchrist et al 2014).

In 2012, *E. bangladeshi* was detected in 4.5% (22 out of 140) of samples in South Africa, marking the first known instance of this species being reported outside of Asia. The potential pathogenicity of this newly identified *E. bangladeshi* species remains uncertain. Interestingly,

the prevalence of *E. bangladeshi* was found to be similar in both diarrhoeal and non-diarrhoeal samples collected from South Africa (Ngobeni et al. 2017). Further investigation is necessary, particularly examining the interactions of *E. bangladeshi* with other co-infecting enteric pathogens, in order to establish its pathogenicity conclusively.

Overall, the epidemiology of *E. bangladeshi* is poorly understood, with limited studies conducted to investigate its incidence.

2.3. Giardiasis

Giardia infection is a highly prevalent intestinal protozoan infection worldwide, making it the most frequent of its kind. It is estimated that approximately 280 million cases of *Giardia* infection occur each year. In resource-poor countries, this parasitic infection alone is responsible for causing around 2.5 million cases of diarrhoea annually (Laishram et al. 2012). In these economically disadvantaged nations, *Giardia* infection is commonly acquired during early infancy, and its prevalence reaches its highest point in children under the age of 10, with rates of up to 30%. In addition to causing diarrhoea, *Giardia* infection in children residing in these countries can lead to stunted long-term growth and cognitive function impairment (Laishram et al. 2012). Due to its significant prevalence in impoverished and underdeveloped communities, giardiasis was included in the World Health Organization's 'Neglected Diseases Initiative' in 2004.

G. lamblia is a species complex comprised of diverse organisms, categorized into distinct genetic groups called "assemblages" (A to G), each with specific host preferences. Human infections primarily stem from assemblages A and B (Laishram et al. 2012).

2.3.1. Structural organization of *Giardia*

Giardia possesses a complex genome comprising approximately 12 million base pairs of DNA with a GC content of 46%. This protozoan demonstrates the ability to undergo antigenic variation of its cysteine-rich surface proteins through a palmitoylated process. This variation occurs not only during human infection but also throughout the encystation and excystation cycles (Adam 2001).

Antigenic variation serves multiple purposes for *Giardia*, including protection against intestinal proteases, maintaining stability in oxygen-rich environments, and facilitating

adaptation to various hosts. However, the clinical significance of antigenic variation of *Giardia* remains unknown.

Cysts, ranging in length from 10 to 20 micrometres, play a significant role in the transmission of *Giardia*. These cysts exhibit relative resistance to chlorination and ozonolysis, enabling them to survive in cold surface water for several weeks to months. Certain environmental conditions, such as cholesterol starvation followed by an alkaline pH and an excess of bile salts, trigger a series of changes. These changes lead to the down-regulation of genes specific to the trophozoite form and result in dipeptidyl peptidase IV-associated proteolysis. As a response to these environmental stimuli, specific encystment vesicles are formed, and there is a transcription and secretion of cysteine-rich cyst wall proteins CWP-1 and CWP-2 into these vesicles. The process of cyst wall formation involves the regulation of Ca2+-dependent degranulation of encystation-specific vesicles by a protein specific to the granules (Adam 2001).

The excystation process is triggered by environmental cues, including the presence of gastric acid and pancreatic enzymes. This activation involves the activation of cysteine proteases and protein kinase A . Excystation leads to the emergence of a single trophozoite that carries four nuclei. This trophozoite undergoes two rounds of division, resulting in the formation of four daughter trophozoites. These trophozoites multiply and establish colonies in the upper part of the small intestine using a ventral sucking disk.

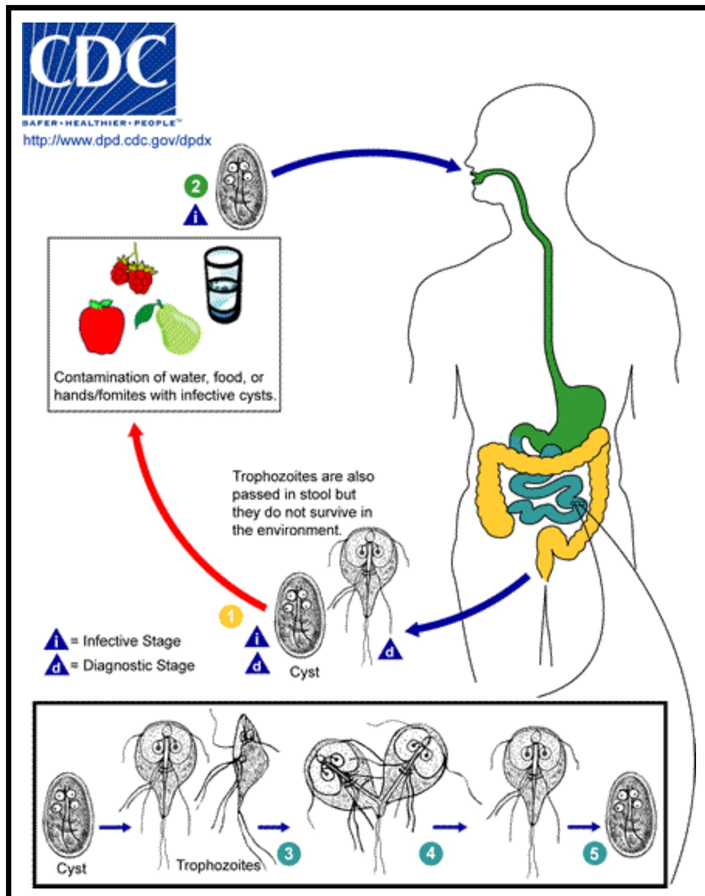
2.3.2. Pathophysiology

The life cycle of *G. lamblia* consists of two main stages: an infectious cyst and a proliferating trophozoite. Transmission of the infection occurs through the faecal-oral route, typically by ingesting *Giardia* cysts present in contaminated water or food. Person-to-person transmission is also possible, and animal-to-person transmission occurs rarely. Even a low number of 10 cysts can lead to a symptomatic infection when ingested.

Following ingestion, excystation takes place in the duodenum. It is believed that this process is initiated by the presence of gastric acid and subsequent exposure to bile and pancreatic proteases in the duodenum. Excystation results in the release of two motile trophozoites, which is made possible by prior nuclear division during the maturation of the cyst **(Figure 2.6).**

Clinical symptoms arise during the trophozoite stage due to intestinal mucosal damage, including brush border microvilli shortening (with or without villous atrophy), disaccharidase deficiency, immune response-induced increased intestinal permeability with anion and fluid hypersecretion, modification of intestinal flora as a potential stimulatory factor for pathogenicity, intestinal barrier dysfunction, and enterocyte apoptosis. The intestinal damage is believed to be caused by cysteine proteases secreted by the trophozoite. Trophozoites primarily reside in the proximal small intestine, specifically the duodenum and jejunum where they attach to enterocytes using a ventral sucking disc (Adam 2001). Although rare, there have been reports of trophozoites being confined to the terminal ileum as well.

These parasites exhibit the characteristic behaviour of being extracellular and do not invade the cells lining the small intestine. Instead, they attach themselves to the microvilli within the intestinal lumen and undergo proliferation. Trophozoites replicate through binary fission, with a doubling time of 9 to 12 hours. Eventually, detached trophozoites transit through the small intestine into the colon, where they undergo encystation. The cyst wall provides protection against environmental factors, allowing the parasite to survive for several weeks to a month in moist and cool environments, such as water at 4°C. Cysts are expelled through faeces and are immediately infectious, playing a crucial role in disease transmission (Adam 2001).



GIARDIASIS LIFE CYCLE

Causal Agent: *Giardia intestinalis* is a protozoan flagellate (Diplomonadida). This protozoan was initially named *Cercomonas intestinalis* by Lambl in 1859. It was renamed *Giardia lamblia* by Stiles in 1915 in honor of Professor A. Giard of Paris and Dr. F. Lambl of Prague. However, many consider the name, *Giardia intestinalis*, to be the correct name for this protozoan. The International Commission on Zoological Nomenclature is reviewing this issue.

Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in the feces (diagnostic stages) 1 . The cysts are hardy and can survive several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food, or by the fecal-oral route (hands or fomites) 2 . In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) 3 . Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk 4 . Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in nondiarrheal feces 5 . Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible. While animals are infected with *Giardia*, their importance as a reservoir is unclear.

Life cycle image and information courtesy of DPDx.

Figure 2.6 Life cycle of *G. lamblia*.

2.3.3. Host Response and Immunity

The immune system plays a crucial role in eliminating and defending against *Giardia* infections through host immunity, which comprises both humoral and cellular components. Research has revealed that antibodies such as IgM and IgG, in conjunction with complement, possess the ability to effectively destroy *Giardia* trophozoites. Nevertheless, within the intestinal lumen where trophozoites reside, secretory IgA antibodies assume a significant role in regulating *Giardia* by binding to trophozoites. This interaction impedes the ability of the parasite to adhere to intestinal epithelial cells, thereby preventing further colonization and infection (Huang and White, 2006).

The lack of secretory IgA has been linked to the inability to effectively eliminate *Giardia* infection in humans and is connected to the development of chronic giardiasis. In studies conducted on mice, it has been found that IL-6 plays a significant role in the initial control of giardiasis, and infection with *G. lamblia* can induce both local (secretory) and systemic antibody responses (Char et al. 1993; Zhou et al. 2003).

Giardia infections cannot be cleared by mice lacking B cells and T cells (Huang and White, 2006). Similarly, individuals with antibody deficits (such as common variable immunodeficiency and X-linked agammaglobulinemia) or reduced gastric acidity (due to previous gastric surgery) are more prone to developing giardiasis. In infected patients, symptoms may include persistent diarrhoea, malabsorption, and distinct histological abnormalities in the small intestine. These abnormalities are characterized by sprue like lesions with pronounced villi flattening, crypt hypertrophy, and dense infiltration of mononuclear cells in the submucosa (Huang and White, 2006).

2.3.4. Clinical Manifestations

Giardiasis can lead to various outcomes depending on the susceptibility of the host and the virulence of the genotype of the pathogen. These outcomes include asymptomatic illness, acute self-limiting diarrhoea, or chronic diarrhoea and malabsorption (Huang and White, 2006). Infection can occur by ingesting as few as 10 to 25 *Giardia* cysts. Among individuals who ingest these cysts, approximately 5% to 15% will pass cysts without experiencing any symptoms, 25% to 50% will develop acute self-limiting diarrhoea, and 35% to 70% will show no signs of infection.

The incubation period for giardiasis typically ranges from 7 to 14 days. Common symptoms of giardiasis include diarrhoea (89% of cases), malaise (84%), flatulence (74%), foul-smelling and greasy stools (72%), abdominal cramps (70%), bloating (69%), nausea (68%), anorexia (64%), and weight loss (64%) (Huang and White, 2006). There are also less frequently reported symptoms such as urticaria (9%), constipation (9%), reactive arthritis, biliary tract disease, and gastric infection. Typically, the white blood cell count remains within the normal range, and there is no presence of eosinophilia. Individuals may describe their bowel movements as having a watery or greasy consistency with a strong, unpleasant odour. The presence of visible blood, pus, or mucus is usually not observed (Huang and White, 2006). There is ongoing debate regarding the detrimental impact of chronic giardiasis on growth and development. Some studies indicate that prolonged episodes of giardiasis in children with pre-existing poor nutrition can have adverse effects, including impaired growth, compromised intestinal permeability, lower weight and height for their age, and reduced cognitive function when compared to healthy children unaffected by giardiasis.

Malabsorption can occur as a consequence of chronic diarrhoea caused by giardiasis. In children, symptomatic giardiasis can result in steatorrhea and impede the absorption of vital nutrients like vitamin A, B12, protein, D-xylose, and iron (Huang and White, 2006). Lactase deficiency, observed in 20% to 40% of cases following a *Giardia* infection, may persist for several weeks even after treatment (Welsh et al. 1984).

2.3.5. Diagnosis and diagnostic studies

The definitive diagnosis of *Giardia* infection involves detecting *Giardia* trophozoites or cysts in stool samples through microscopic examination of wet mounts and concentrated samples. Stool specimens can be preserved using polyvinyl alcohol and/or 10% formalin for permanent staining with trichrome or iron hematoxylin stains. Since the parasites may be excreted irregularly, examining a single stool specimen has a sensitivity of only 50 to 75%. However, the sensitivity increases to over 90% when three consecutive specimens are collected every two to three days (Huang and White, 2006).

The recommended diagnostic tests for *Giardia* infection include Direct Fluorescent Antibody (DFA) tests, which identify intact organisms, and Enzyme-Linked Immunosorbent Assays (ELISA), which detect soluble antigens in stool samples. In certain developed countries, the utilization of multiplex real-time Polymerase Chain Reaction (PCR) assays for detecting

Giardia and other gastrointestinal protozoan pathogens has gained popularity, replacing microscopy as a routine diagnostic tool (Huang and White, 2006).

Real-time PCR has demonstrated a sensitivity of 98% and a specificity of 100%. When applied to stool samples, PCR assays can identify specific genes of the parasite even at concentrations as low as 10 parasites per 100 Maximum Contaminant Level (MCL). This makes PCR a valuable screening tool for assessing the quality of water supplies (Huang and White, 2006).

2.3.6. Treatment

The treatment of symptomatic giardiasis has involved the use of various drugs. The recommended drugs of choice include metronidazole (Flagyl) administered orally in three doses of 15mg/kg/day (up to a maximum of 750mg/day) for a duration of 5 to 10 days, tinidazole (Tindamax, Fasigyn, Simplotan, Sporinex) administered as a single oral dose of 50mg/kg (up to a maximum of 2g), and nitazoxanide (Alina, Allpar, Adonid, Annita, Daxon, Dexidex, Nizonide) administered orally twice a day at a dose of 7.5mg/kg for a period of 3 days. Tinidazole, due to its single-dose administration and fewer side effects, is considered preferable over metronidazole and nitazoxanide for children aged 3 years and above. Side effects of tinidazole may include metallic taste, nausea, headaches, and, in rare cases, hepatitis and cholangitis (Gardner and Hill 2001).

Additional medications that can be utilized for the treatment of giardiasis include:

- Albendazole (Albenza, Valbazen, Zentel): Administered orally once daily for 5 days at a dosage of 10 to 15mg/kg (up to a maximum of 400mg).
- Mebendazole (Vermox): Taken orally twice a day for 3 days at a dosage of 100mg.
- Ornidazole (Xynor): Administered as a single oral dose of 20 to 40mg/kg (up to a maximum of 2g).
- Secnidazole (Flagentyl, Sindose, Secnil, Solosec): Taken orally as a single dose of 30mg/kg (up to a maximum of 2g).
- Paromomycin (Catenulin, Aminosidine): Administered orally three times a day for 10 days at a dosage of 10mg/kg (up to a maximum of 500mg).
- Mepacrine/Quinacrine (Atabine): Taken orally three times a day for 5 days at a dosage of 2mg/kg (up to a maximum of 100mg).

- Furazolidone (Furoxone, Dependal-M): Taken orally four times a day for 7 to 10 days at a dosage of 2mg/kg (up to a maximum of 100mg).
- Albendazole, mebendazole, and metronidazole exhibit similar efficacy in treating giardiasis; however, albendazole and mebendazole are associated with fewer side effects compared to metronidazole or tinidazole.

These medications provide alternative options for treating giardiasis (Gardner and Hill 2001).

2.3.7. Prevention

The primary preventive measure that holds utmost importance is maintaining meticulous hand hygiene using soap and water. This practice is particularly critical for staff members working in childcare centres, especially after using the toilet or changing diapers. It is essential to exclude infected children with diarrhoea from childcare centres until their symptoms have subsided. To minimize the transmission of giardiasis, it is crucial to implement training programs that enhance the awareness of food handlers. Lastly, it is vital to ensure proper treatment of individuals displaying symptoms to effectively manage the condition (Huang and White, 2006).

2.3.8. Epidemiology:

Giardiasis, a common enteric protozoal infection, is widespread globally. It affects approximately 2% of adults and 8% of children in developed nations, while around 33% of individuals in developing countries have experienced giardiasis. In the United States, it is estimated that there are about 1.2 million cases, but the majority of them go undetected since carriers often do not display any symptoms. According to data from the Centers for Disease Control and Prevention (CDC), 15,223 cases were reported in 2012.

The demographic most significantly impacted was the age group of children aged 0 to 4 years, and the northwest region of the United States reported the highest proportion of cases. The highest occurrence of infections typically happens during the late summer and early autumn, primarily due to outdoor water-related activities. Back in 1988, the World Health Organization (WHO) approximated an annual infection rate of approximately 280 million individuals in Asia, Africa, and Latin America caused by various species of *Giardia*. Giardiasis is acknowledged as a prominent source of gastrointestinal illness among travelers visiting the United States, Canada, and Europe (Harvey et al. 2013; Boggild, 2014; Schlagenhauf et al.

2015). In a comprehensive study involving 147 pediatric patients in the United States who experienced acute non-dysenteric diarrhoea, giardiasis was identified as the cause in 15 percent of cases, ranking second after rotavirus.

2.3.9. Giardiasis in India

Giardiasis has been found to be widespread across India, according to multiple epidemiological studies. In northern India, community-based studies have shown prevalence rates ranging from 5.5% to 70%, with the highest rates reported among a low socioeconomic group in Chandigarh (Laishram et al. 2012). Children were particularly affected, as high rates of both asymptomatic cyst passage and diarrhoea associated with giardiasis were identified in this group (Laishram et al. 2012). **Table 2.7** provides a summary of the prevalence rates of giardiasis observed in various studies conducted in India.

Table 2.7 : Prevalence rates of giardiasis observed in various studies in India

Site of study	*n*	Year	Population	% Prevalence
Northern India				
Amritsar	150	1995	Children <3 y with chronic diarrhea	4
Chandigarh	550	2004	Low socioeconomic status	6
Chandigarh	600	2005	General population	5.5
Chandigarh	970	1991	Low socioeconomic status	69.5
Chandigarh	82,667	2000	Outpatients	4–9
Chandigarh	120	1994	Infants with intractable diarrhea	6
Delhi	175	2008	Children with persistent diarrhea	20
Delhi	127	2002	Children with diarrhea	11
Delhi	100	2008	Adult and children with malabsorption	24
Delhi	939	2002	Urban slum dwellers	8.4
Lucknow	1,071	2007	Urban and rural population	22
Lucknow	1,061	1997	Pre-school slum children	32.9
Punjab	–	1986	Pre-school children	35.1
Srinagar	514	2007	School children	7.2
Southern India				
Bangalore	361	1990	Children with diarrhea	8–10 (<6 m–2.1)
Pune	76	1991	Children <5 y with diarrhea	7.9
Chennai	324	2002	Rural (*n*=125) and urban (*n*=199) population	16 (rural), 22.6 (urban)
Karnataka	10,000	1998	Adults	37.1
Karnataka	1,020	1989	Adult and children, healthy	2.5
Vellore	78	1998	Asymptomatic rural population	53.8
Vellore	452	2009	Children in urban slum	22.9
Eastern India				
Varanasi	2,095	1999	Patients with acute diarrhea	1.7
Bihar	326	1996	Rural and semi-urban	28.2
Kolkata	383	1984	Under five children with diarrhea	0.4
Kolkata	1,103	2009	Children and adults with diarrhea	13.3
Kolkata	2,519	2010	Hospitalized patients with diarrhea	11.2
Sikkim	2,559	1970	General population	5.9

(Source: Laishram et al. 2012; DOI 10.1007/s12664-012-0161-9)

2.4. *Cryptosporidiosis*

Cryptosporidium, a protozoan parasite, is a major cause of diarrhoeal illness in both humans and animals worldwide. With over 40 recognized species, some of which affect humans, the most commonly detected ones are *Cryptosporidium hominis* and *Cryptosporidium parvum*. Other less frequently identified species include *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium ubiquitum*, *Cryptosporidium cuniculus*, *Cryptosporidium muris*, and *Cryptosporidium viatorum*. The infection and diseases

associated with Cryptosporidium parasites have significant impacts on health, welfare, and the economy.

Protozoal infections are responsible for over 58 million cases of diarrhoea detected annually in children. These infections, particularly those caused by waterborne pathogens like *Cryptosporidium* and *Giardia*, have been recognized as part of the World Health Organization's "Neglected Disease Initiative". While Cryptosporidium infections typically result in acute self-limiting gastroenteritis in individuals with a healthy immune system, immunocompromised individuals may experience chronic and life-threatening diarrhoeal disease as a consequence (Innes et al. 2020).

Neonates are particularly vulnerable to infections because their immune system is not fully developed. They can easily contract infections by consuming even small amounts of the parasite's oocysts. Each year, diarrhoeal diseases are responsible for causing approximately 1.6 million deaths globally. Shockingly, one-third of these deaths occur in children under the age of 5 due to contaminated drinking water and inadequate hygiene practices. In developing countries, *Cryptosporidium* is responsible for up to 20% of all cases of diarrhoea in children, and it poses life-threatening complications for individuals with HIV(Helmy and Hafez 2022). Furthermore, *Cryptosporidium* is a major cause of more than 8 million cases of foodborne illnesses worldwide annually. *Cryptosporidiosis* predominantly impacts individuals residing in rural areas and urban slums, where the likelihood of disease transmission and spread is significantly higher (Helmy and Hafez 2022).

The success of *Cryptosporidium* spp. as parasites can be attributed to several factors, including their ability to infect a wide range of hosts, their capacity to produce a large number of oocysts in infected individuals, their transmission through water, and their ability to cause infection even at low doses. Recent modelling studies have underscored the substantial risk of infection, with evidence suggesting that as few as one oocyst can lead to successful infection.

2.4.1. Life Cycle and Developmental Stages of *Cryptosporidium:*

Cryptosporidium sp. oocysts are tiny, measuring 4-6 µm in diameter, and have a spherical-to-ovoid shape. The entire life cycle of *Cryptosporidium* occurs within a single host and involves both asexual and sexual stages. Tzipori and Ward described six key developmental stages in this cycle: excystation, merogony, gametogony, fertilization and zygote development,

formation of a durable oocyst wall, and sporogony leading to either type II meronts or another round of type I meronts. Type II meronts produce four merozoites that can either develop into microgametes or macrogamonts (Mekonnen et al. 2016) (**Fig 2.7)**.

The fusion of gamonts through fertilization gives rise to a zygote that undergoes further development, eventually forming an oocyst containing four sporozoites. The resulting oocysts can be classified into two types: thick-walled oocysts, which are eliminated from the body through faeces, and thin-walled oocysts, which remain within the intestinal tract, leading to autoinfection. This mechanism potentially elucidates the persistence of infection in individuals with AIDS, even in the absence of subsequent exposure to oocysts (Mekonnen et al. 2016).

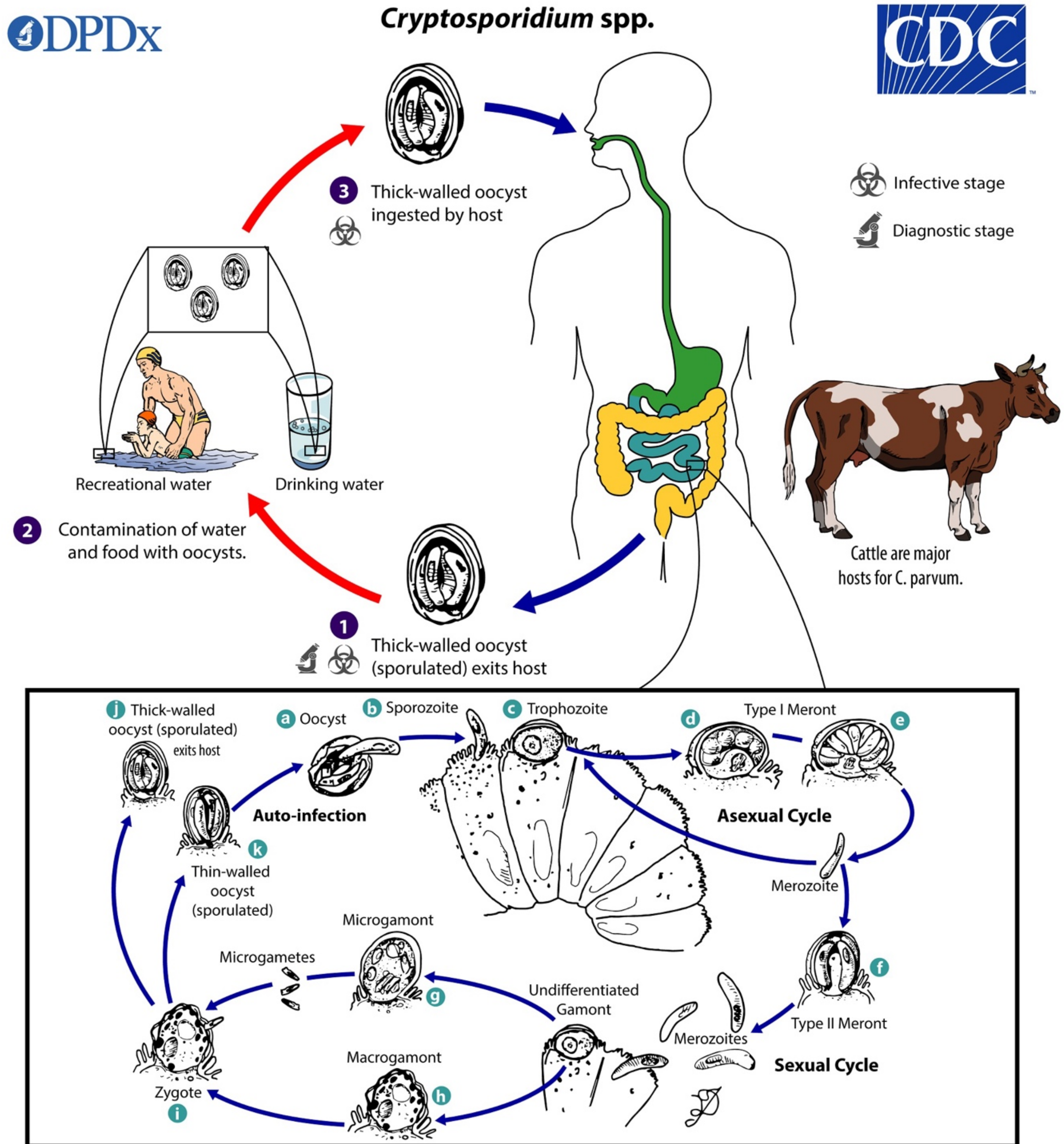


Fig 2.7 Life Cycle and Developmental Stages of *Cryptosporidium*

2.4.2. Mode of Transmission:

Cryptosporidium is widely distributed in nature and is primarily transmitted through the two-layered thick-walled oocysts. Even a low dose of 10-100 oocysts can lead to infection transmission. The parasite can be transmitted through both zoonotic and non-zoonotic means. *Cryptosporidium* oocysts exhibit remarkable resilience and can withstand diverse environmental conditions. They can survive in water and soil for extended periods, facilitated by favourable moisture and cold temperatures. Due to their small size, they can be easily transported over long distances through the air or rapidly flushed into water sources (Vanathy et al. 2017).

2.4.2.1. Zoonotic transmission

Zoonotic transmission played a significant role in the early emergence of *Cryptosporidium* outbreaks. The initial case was reported in a child residing on a farm where cattle rearing took place. Factors such as calves, lambs, animal faeces, and inadequate hand hygiene were identified as contributors to the zoonotic spread of the disease (Vanathy et al. 2017).

2.4.2.2. Nonzoonotic transmission

A. Waterborne transmission

Waterborne transmission is a major route of *Cryptosporidium* infection. In 1993, an outbreak occurred primarily among immunocompromised patients, resulting in around 403,000 cases, with 5,000 confirmed cases and 100 fatalities. Due to its potential for water contamination, *Cryptosporidium* was categorized as a level B pathogen by the CDC and NIH. It can be found in various water sources, including surface water, groundwater, treated and untreated drinking water, as well as recreational water like swimming pools and lakes. Contamination of these water systems can happen through the introduction of human and animal excreta used as crop fertilizers or through contaminated sewage entering the water distribution system. Water is a frequent source of infection primarily because oocysts, which are responsible for causing infections, have a high resistance to many chemical purification methods like chlorination. Nevertheless, their presence can be minimized through various treatment methods such as

coagulation, filtration, and sedimentation. Therefore, controlling these infections requires implementing a combination of treatment systems (Vanathy et al. 2017).

B. Foodborne transmission

Food and food products can also be a potential source of infection, leading to reported cases of foodborne outbreaks. Among the sources linked to such outbreaks are apple cider, chicken salad, milk, general food items, commercially packaged oysters with a seafood-like nature, and raw vegetables (Vanathy et al. 2017).

C. Other sources

Nosocomial transmission can occur through interactions with hospital personnel, as well as in day care centres. Mechanical transmission of infections can happen through contact with soil or insects such as cockroaches and houseflies. Oocysts, the infective stage of certain parasites, have been detected in sputum and bronchial aspirates. Additionally, this pathogen can cause traveller's diarrhoea (Vanathy et al. 2017).

2.4.3. Pathogenicity and virulence factor:

The infectious form of *Cryptosporidium* is the small sporulated thick-walled oocyst, measuring approximately 4-6 µm in diameter. It is noteworthy that even a minimal quantity of ten oocysts can lead to infection. The entire life cycle of *Cryptosporidium* takes place within a single host, specifically on the luminal surface of the intestinal epithelium, where it remains in the intracellular extracytoplasmic region of the host cell (Vanathy et al. 2017). Following the ingestion of the oocyst, attachment to the epithelium occurs through the assistance of glycoproteins such as gp900, gp60, and the circumsporozoite ligand. Subsequently, motile sporozoites are released within the intestine. Utilizing specialized organelles called rhoptries and micronemes, they penetrate the host cell and establish parasitophorous vacuoles. The life cycle involves both asexual reproduction (merogony) and sexual reproduction (gametogony). Thick-walled oocysts are produced and excreted in the faeces, while thin-walled oocysts facilitate autoinfection (Vanathy et al. 2017).

The changes in the intestinal epithelium caused by the parasite result in a reduction in villous length and an increase in crypt length. Additionally, the parasite induces the production of inflammatory mediators like interferon-gamma (INF-β), interleukin (IL)-8, and tumour

necrosis factor (TNF), which then stimulate the release of soluble factors. These factors enhance chloride and water secretion while reducing sodium absorption, leading to the development of osmotic diarrhoea.

2.4.3.1. Virulence factors

Virulence factors are responsible for inducing damage to the host cells. This detrimental effect arises from the interaction between the host and the parasite. Multiple virulence factors play a role in processes such as excystation, epithelium attachment, gliding motility, invasion, intracellular survival, and causing damage to host cells (refer to **Table 2.8** for details) **(**Vanathy et al. 2017).

Table 2.8. Virulence Factors of *Cryptosporidium* spp.

Virulence factors	Functions
Serine proteases, aminopeptidase	Excystation
Circumsporozoite such as glycoprotein, gp900, gp60/40/15, P30, Cp47	Adhesion
P23, TRAP-C1, CPS500, CpMIC1	Adhesion, locomotion
Cp2, secretory phospholipase, Cpa135, CpSUB	Invasion
Hemolysin H4	Membrane lysis
CpABC	Nutrient transport
CpATPase2	Biomembrane heavy metal transporter
CpATPase3	Biomembrane ion or phospholipid transporter
HSP70, HSP90	Stress protection
Cysteine protease	Immune/cytokine modulation
Acetyl Coa synthetase	Fatty acid metabolism

(Source: Vanathy et al. 2017. doi:10.4103/tp.TP_25_17)

2.4.4. Immunity to *Cryptosporidium* infection

Both innate and adaptive immune responses contribute to immunity against *Cryptosporidium* infections. Innate immunity involves natural killer cells, dendritic cells, macrophages, chemokines, toll-like receptors, and nitric oxide synthase. Adaptive immunity primarily relies on T- and B-cell responses. INF-β plays a crucial role in innate immunity by activating macrophages to produce inducible nitric oxide synthase, leading to the generation of nitric oxide and activation of the stress signalling pathway. Adaptive immunity relies on the crucial

involvement of T- and B-cells. Specifically, Th1 CD4 helper cells combat intracellular parasites by generating INF-β, IL-2, IL-12, and TNF, while Th2 helper cells aid in the elimination of parasites. It has been observed that individuals with very low CD4 counts experience more severe infections compared to those with CD4 counts exceeding 200 cells/mm3. The significance of humoral immunity in AIDS patients with Cryptosporidium infection remains uncertain. However, these individuals exhibit elevated levels of IgG/IgA and mucosal IgA (Vanathy et al. 2017).

2.4.5. Clinical presentation:

The severity of an illness typically relies on the immune status of the individual, leading to a range of manifestations that can span from asymptomatic to extremely severe or fulminant disease.

2.4.5.1. In immunocompetent individuals

Both individuals with a fully functioning immune system and those with compromised immune systems can exhibit an asymptomatic presentation. In individuals with a healthy immune status, the condition is self-limiting. Symptoms may include 5-10 episodes of watery diarrhoea per day accompanied by mucus flecks. Less common manifestations include nausea, abdominal cramps, low-grade fever, and anorexia. Typically, the illness resolves within a span of two weeks. In infants with chronic cryptosporidiosis, failure to thrive may occur, while malnourished children may experience stunted growth and respiratory tract involvement (Vanathy et al. 2017).

2.4.5.2. In immunocompromised individuals:

Human immunodeficiency virus individuals: The condition manifests in various ways, ranging from no noticeable symptoms to severe and rapid-onset illness. The symptoms can persist for several months, with individuals experiencing the passage of 3–6 liters of persistent watery stools per day. This excessive amount of stool is primarily observed in individuals with a CD4 count below 200 cells/mm3, and there have been reports of individuals passing as much as 17 liters of watery stool in a day. Studies have identified four distinct presentations of the illness: chronic diarrhoea (36% of cases), cholera-like illness requiring intravenous rehydration

therapy (33% of cases), transient diarrhoea (15% of cases), and intermittent diarrhoeal illness (15% of cases). Over time, the symptoms tend to worsen, leading to a decline in the average survival rate of affected individuals (Vanathy et al. 2017).

2.4.6. Laboratory diagnosis

Traditionally, the routine diagnosis of cryptosporidiosis in many countries has relied on the microscopic identification of oocysts through the staining of fecal smears. Various concentration techniques involving flotation and sedimentation principles have been extensively employed, utilizing solutions like sucrose, salt, zinc sulfate, and formol-ether. The staining methods most frequently utilized include the modified Ziehl-Neelsen acid-fast stain and the modified Kinyoun's acid-fast stain. Differential staining methods, including safranin-methylene blue, have been commonly employed in the field. Another technique involves negative staining with stains like nigrosin, light green, merbromide, and malachite green, which effectively stains yeasts and bacteria while leaving oocysts unstained. However, these techniques have limitations. Despite their ease of use and affordability, they are unable to differentiate between different species of *Cryptosporidium* (Rossle and Latif, 2013).

2.4.7. Treatment

Nitazoxanide (Alinia), an anti-protozoal agent, was the sole drug approved by the US Food and Drug Administration in 2006 for the treatment of cryptosporidiosis in children and immunocompetent adults. However, its effectiveness is dependent on a suitable immune response and, as a result, it is not effective in treating individuals with compromised immune systems.

The utilization of highly active antiretroviral therapy (HAART) has significantly enhanced recovery and survival rates among immunocompromised individuals, leading to notable increases in CD4+ T-lymphocyte counts. Furthermore, the incorporation of protease inhibitors into HAART has provided additional advantages, as these inhibitors directly disrupt the parasite's life cycle, offering further therapeutic benefits.

The treatment of cryptosporidiosis often involves a combination of drugs. Paromomycin, an aminocyclitol antibiotic derived from Streptomyces, has shown effectiveness in combination with protease inhibitors or recombinant IL-12. When used together, these drug combinations

can enhance the efficiency of paromomycin in treating cryptosporidiosis (Rossle and Latif, 2013).

2.4.8. Epidemiology

Cryptosporidium is a known cause of diarrhoea in individuals with both healthy and weakened immune systems. The primary species responsible for human cryptosporidiosis are *C. hominis* and *C. parvum*, although their prevalence varies across different geographic areas. In addition to these two species, other *Cryptosporidium* species like *C. felis, C. muris,* and *C. meleagridis* have been documented to infect humans and contribute to cases of *Cryptosporidium*-related infections. *Cryptosporidium* is commonly found in developing countries, particularly affecting adults with HIV infection and children. The prevalence of *Cryptosporidium* infection among children with diarrhoea in underdeveloped countries like Brazil, Venezuela, Indonesia, Thailand, South Africa, Ghana, India, and Bangladesh ranges from 3% to 13%. However, in developed regions such as Britain, the United States, Canada, Australia, and Denmark, it constitutes only 1-4% of cases of childhood diarrhoea (Vanathy et al. 2017*,* Mahmoudi et al 2017).

2.4.9. Cryptosporidiosis in India

In India, the prevalence rate of cryptosporidiosis has been documented as ranging from 4% to 13%. Within South India, approximately 40% of children residing in semi-urban slums experience recurrent episodes of cryptosporidiosis and prolonged oocysts shedding of the parasite. Similarly, in Eastern India, children living in peri-urban regions exhibit a high prevalence of being asymptomatic carriers of the infection. These studies establish a clear association between inadequate sanitation practices and the occurrence of *Cryptosporidium* infection (Mahmoudi et al 2017).

2.4.10. Cryptosporidiosis in children

Cryptosporidium is known to cause nutritional deficiencies, leading to considerable illness and even death, particularly among children and individuals with weakened immune systems. The main line of defence against cryptosporidiosis is the activation of cell-mediated immune responses. Because children have immature immune systems, they are highly susceptible to

infection. Malnutrition and certain childhood infections like measles contribute to the weakened immune state in children, making them more prone to becoming immunocompromised (Vanathy et al. 2017).

Several studies conducted in India have documented the presence of *Cryptosporidium* spp. in children experiencing diarrhoea, with reported prevalence rates ranging from 1.1% to 18.9%. Additionally, studies have found *Cryptosporidium* spp. in asymptomatic children, with prevalence rates ranging from 0% to 3%. Furthermore, some studies have reported up to 9.8% positivity for *Cryptosporidium* spp. in asymptomatic children, while symptomatic children exhibited a higher positivity rate of 13.1% (Vanathy et al. 2017).

CHAPTER III

HOSPITAL-BASED SURVEILLANCE OF COMMON DIARRHOEAGENIC ENTERIC PARASITES IN KOLKATA

Hospital-based surveillance of common diarrhoeagenic enteric parasites in Kolkata

3.1. Background

Diarrhoea is responsible for an estimated 15% of all child deaths in children under the age of five, with higher rates observed in specific regions such as approximately 31% in South East Asia and 25% in Africa. Furthermore, it remains a significant cause of mortality among adults in these countries. Among children under the age of five, it ranks as the second most common cause of death due to infectious diseases, while also being a significant contributor to adult mortality in these nations. The prevalence of diarrheal diseases is closely linked to the economic condition of a country and has multifaceted impacts on its economy, particularly by compromising the health and productivity of its workforce. Diarrheal diseases continue to prevail as a significant cause of mortality in regions like South Asia and sub-Saharan Africa, consistently ranking among the top four factors that contribute to years of life lost. Diarrhoea manifests in three distinct clinical syndromes: acute watery diarrhoea, dysentery, and persistent diarrhoea. Each syndrome represents a different underlying cause and necessitates specific treatment approaches. In addition to bacteria and viruses, parasites play a significant role in causing diarrhoea, particularly in developing countries.

Enteric parasites continue to play a significant role in the occurrence of diarrhoeal diseases, affecting populations in both developing and developed nations. Despite notable advancements in reducing overall mortality rates, including those specifically associated with diarrhoea, these diseases persist and result in a significant number of deaths among children under the age of five worldwide. It is important to note that parasitic infections can sometimes be overlooked, leading to an uncertain understanding of their causes after a certain period of time. Diarrhoea-causing parasitic protozoa found in the intestinal tract include *E. histolytica*, *G. lamblia*, and *Cryptosporidium* species. These organisms are responsible for amoebiasis, giardiasis, and cryptosporidiosis and are commonly associated with diarrheal illnesses. In developing regions such as sub-Saharan Africa and South Asia, these three parasites are frequently identified as causative agents in cases of diarrhoea. On the other hand, enteric parasites like Blastocystis spp. and *Dientamoeba fragilis* appear to be more prevalent in developed countries (Nair et al. 2010, Fletcher et al. 2011 Roberts et al. 2011; Fletcher et al. 2012).

In Kolkata, our laboratory has observed a significant presence of parasitic infections based on recent hospital data on diarrheal morbidity. Notably, asymptomatic infections are also prevalent in this population. According to studies conducted by Mukherjee et al. in 2009 and Das et al. in 2014, there is a substantial prevalence of *Giardia lamblia, Entamoeba histolytica* and *Cryptosporidium* spp. in this region. Nevertheless, these studies alone are insufficient for accurately estimating the burden of parasitic infections and understanding the trends of diarrheal parasitic infections. Therefore, it is highly recommended to properly identify and characterise the parasites to enhance prevention and control measures for parasitic diarrheal incidences. Comprehensive efforts in identifying and understanding the specific parasites involved are crucial for the effective management and mitigation of parasitic-related diarrhoea.

India is recognized as a country with a high incidence of enteric parasitic infections. Kolkata, a densely populated city in eastern India and Southeast Asia, experiences significant immigration and emigration of individuals from diverse cultural backgrounds and varying socioeconomic conditions. These factors contribute to a favourable climate for the proliferation of enteric pathogens, thereby increasing the likelihood of encountering numerous opportunistic enteric parasites in this region. However, there is a scarcity of information regarding the diversity of *Entamoeba* species infecting individuals in this area. Kolkata continues to witness a significant number of patients afflicted by diarrhoea, which remains a pressing concern not only for the city but also for eastern India as a whole, leading to indirect economic implications. The Beliaghata Infectious Disease Hospital and B. C. Roy Children Hospital consistently observe a steady influx of diarrhoeal cases. Fortunately, the presence of a well-equipped parasitology laboratory at the ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED) has facilitated the expansion of hospital surveillance for parasitic diarrhoea. This has, in turn, enabled the undertaking of the present study, aiming to enhance our understanding of the issue.

The objective of this study was to assess the prevalence of common enteric parasites among patients suffering from diarrhoea in Kolkata and its surrounding areas. Descriptive scientific studies addressing the prevalence and seasonal distribution of *Entamoeba* species, *Cryptosporidium* species, and *Giardia lamblia* in and around Kolkata have not been conducted, thus limiting our comprehensive understanding of their occurrence. Considering the public health importance of intestinal protozoa infections, the objective of the present study was to identify and molecularly characterize intestinal protozoa through active surveillance conducted

at Beliaghata Infectious Disease Hospital and B. C. Roy Children Hospital in Kolkata, India. This study aims to address the aforementioned concerns by providing updated information on the prevalence of parasitic diarrheal diseases, as well as the seasonal patterns of various enteric parasites in the study area.

This chapter focuses on the occurrence of the three most prevalent enteric parasites, namely *G. lamblia, E. histolytica,* and *Cryptosporidium* spp., in Kolkata and its neighbouring regions. The study provides detailed insights into the presence and distribution of these parasites in the area.

3.2. Methodology

3.2.1. Study area and population

This study focused on conducting hospital-based systemic surveillance from March 2017 to February 2020. The aim was to observe patients admitted to Infectious Disease & Beliaghata General Hospital and Dr. B C Roy Post Graduate Institute of Paediatric Sciences Hospital, Kolkata, with complaints of diarrhoea. The target population included patients from various parts of Kolkata and nearby areas. These hospitals are recognized as referral centres for the treatment of diarrheal diseases, in addition to government healthcare facilities. It's important to note that this study excluded cases that did not involve diarrhoea. The study employed a systematic sampling approach to include patients who were admitted to the hospital with complaints of diarrhoea. Specifically, on two randomly selected days each week, every fifth patient presenting with diarrhoea or dysentery but without any associated complaints was enrolled in the study. This sampling process ensured an unbiased representation of patients in terms of their sex and age at the time of selection. Furthermore, faecal samples were collected from all the enrolled patients and subjected to analysis. The faecal samples were collected from the patients admitted to the hospital by trained medical professionals in the presence of attending physicians on the first day of hospitalisation and before antibiotic therapy. Samples were collected in a sterile container with a unique identification number. Once the samples were collected, they were immediately sent to laboratories for testing after written informed consent was obtained from patients.

In addition to the aforementioned surveillance, an epidemiological survey was conducted and documented for the study population. This survey included the stratification of data based on age and sex, as well as the characterization of the nature of parasitic infestations. Furthermore, the study recorded the month-wise prevalence of different parasites. The primary objective of

this systematic study was to assess and reveal the prevailing burden of common enteric parasitic infestations in the Kolkata region and its surrounding areas.

3.2.2. Ethical statement

This study received ethical clearance from the Institutional Human Ethics Committee of the ICMR-National Institute of Cholera and Enteric Diseases (IRB Number: A-1/2015-IEC). Informed consent statements were obtained from all participants, ensuring their voluntary participation in the study. For children, voluntary informed consent was obtained from their caregivers, such as parents or guardians, to ensure ethical considerations were met.

3.2.3. Microscopy

For microscopic analysis, the collected samples were concentrated. Subsequently, the concentrated samples were mixed with 2.5% potassium dichromate for preservation and stored at 4°C. The identification of parasites was primarily conducted using microscopy, utilizing both saline wet mounts and Lugol's iodine wet mounts. The microscopy analysis also included an examination of unpreserved samples. To identify the parasites present in the faecal samples, three distinct techniques were employed. Trophozoites and parasite cysts were identified using iodine wet mount staining. *Cryptosporidium* sp. was detected using modified Kinyoun's Acid fast staining. Lastly, *Giardia* sp. and *Entamoeba* sp. were identified through Trichrome staining. Each of these techniques allowed for specific and accurate identification of the respective parasites in the samples.

3.2.3.1 Concentration method

- The stool sample, consisting of 0.15-0.2 grams for a solid stool or 400 microliters for liquid stool, was transferred into a 50-millilitre conical tube. Normal saline was added and thoroughly mixed to disperse the large mass of stool.
- The entire mixture was then passed through a mesh with a pore size of 40 to 50 microns to remove unwanted debris.
- Afterwards, the filtered stool was subjected to centrifugation at 1500 rpm for 10 minutes, and the resulting supernatant was discarded.
- The concentrated stool pellet obtained from centrifugation was utilized for the preparation of microscopic slides.

A. Iodine wet mount staining

To examine the presence of *Entamoeba* spp. or *Giardia lamblia*, a small amount of fecal sample was mixed with either normal saline or iodine solution. A thin smear of this mixture was then carefully applied onto a clean glass slide and covered with a cover slip. Each slide was examined meticulously using a CX41 phase contrast microscope (Olympus, Tokyo, Japan) with a 40× objective lens to detect the presence of *Entamoeba* spp. or *Giardia lamblia*.

Reagents:

To prepare the reagent, a solution of iodine was made using the following ingredients:

- 5 grams of powdered iodine crystals
- 10 grams of potassium iodide (KI)
- 100 millilitres of distilled water.

First, the potassium iodide was dissolved in the distilled water. Then, the iodine crystals were gradually added to the solution. The resulting mixture was filtered and transferred to an amber-coloured bottle for storage.

B. Trichrome Staining Procedure

Stained faecal films are widely acknowledged as the most effective method for examining stool samples to identify intestinal protozoa. The utilization of permanent stained smears enhances the detection and characterization of cysts and trophozoites, while also providing a lasting record of the encountered protozoa. The stained smear technique used for faecal specimens, known as the Wheatley Trichrome technique, is a modification of Gomori's original staining procedure designed for tissue samples. It offers several advantages over wet mount examinations, as it enables the detection of small protozoa that may have been missed in both unconcentrated and concentrated samples. This rapid and straightforward procedure consistently produces well-stained smears, allowing for clear visualization of intestinal protozoa, human cells, yeast, and artefacts.

Specimen

Typically, the specimens for analysis consist of fresh stool or concentrated fresh stool that is spread onto microscope slides. These slides are then left to air dry naturally or dried using a slide warmer set at a temperature of 60°C.

Reagents

The following solutions were utilized in the process:

1. 70% Ethanol plus iodine: A stock solution was prepared by combining iodine crystals with 70% alcohol until a dark solution was obtained. For use, the stock solution was diluted with 70% alcohol until a dark reddish-brown or strong tea color was achieved.
2. 70% Ethanol (twice)
3. Trichrome Stain: This stain could be obtained commercially (Trichrome stain AB solution, Sigma Aldrich).
4. 90% Acid Ethanol: A mixture of 90% ethanol (99.5 ml) and 0.5 ml of glacial acetic acid.
5. 95% ethanol
6. 100% ethanol (twice)
7. Xylene or xylene substitute (twice)

Procedure

1. The slide underwent a 10-minute immersion in 70% ethanol plus iodine.
2. It was then transferred to 70% ethanol for a duration of 5 minutes.
3. Subsequently, it was placed in a second 70% ethanol solution for 3 minutes.
4. The slide was subjected to Trichrome stain for 10 minutes.
5. Following the staining process, it underwent a brief distaining step in 90% ethanol plus acetic acid, lasting 1 to 3 seconds.
6. Multiple rinses were performed using 100% ethanol.
7. The slide was then exposed to two changes of 100% ethanol, with each change lasting 3 minutes.
8. Next, it was placed in two changes of xylene or xylene substitute for a duration of 10 minutes.
9. Subsequently, the slide was mounted with a coverslip using a mounting medium, such as permount.
10. The resulting smear was examined microscopically utilizing the 100× objective, with a minimum of 200 to 300 oil immersion fields being examined.

Quality Control

As part of each staining run, a control slide containing a preserved specimen of a well-known protozoan, such as *Giardia* spp., is included. When the smear is properly fixed and the staining process is carried out correctly, the cytoplasm of protozoan trophozoites will exhibit a distinct blue-green colour, occasionally with a hint of purple. On the other hand, cysts tend to display a slightly more pronounced purple hue. The nuclei and inclusions (such as chromatid bodies, red blood cells, and bacteria), as well as Charcot-Leyden crystals, exhibit a red colour, occasionally with a touch of purple. Glycogen, when dissolved by solvents, appears as a clear area within the organism. The background material typically stains green, creating a pleasant colour contrast with the protozoa.

C. Kinyoun's Acid fast staining

This staining method proves beneficial in identifying oocysts of coccidian species (such as *Cryptosporidium, Cystoisospora,* and *Cyclospora*) that can be challenging to detect using common stains like trichrome. Unlike the Ziehl-Neelsen Modified Acid-Fast Stain, this technique eliminates the need for heating reagents during the staining process.

Specimen

The concentrated sediment from fresh stool samples was used.

Reagents

1. Pure methanol
2. Acid alcohol solution: 1 ml of sulfuric acid + 99 ml of absolute ethanol. Store at room temperature.
3. Kinyoun's carbol fuchsin: commercially purchased.
4. Commercially obtained methylene blue.

Procedure

The Kinyoun stain is a modified version of the acid-fast stain and is often referred to as the cold method because it does not require heating during the staining procedure. To perform the staining, a thin smear of faeces is first made and fixed with methanol for one minute. Then, the slide is flooded with carbol fuchsin for five minutes, followed by rinsing with 50% ethanol for 3-5 seconds and water. Next, the slide is decolourized using 1% sulfuric acid for two minutes or until no colour runs from the slide. After rinsing with water, the smear is counterstained with methylene blue for one minute. Finally, the slide is examined under a microscope using a 100x oil immersion objective.

Quality Control

In each staining run, a control slide containing *Cryptosporidium* spp. is included. This control slide is derived from a 10% formalin-preserved specimen. When stained, *Cryptosporidium* spp. appears as a pinkish-red colour (oocysts are rounded and measure 4.2 to 5.4 µm in diameter). The background stain, on the other hand, exhibits a consistent and uniform blue colouration.

D. Molecular Screening

The extraction of genomic DNA from the fresh samples was carried out using the QIAamp DNA Stool Mini Kit (Qiagen, USA), following the instructions provided by the manufacturer. Subsequently, the eluted DNA was stored at a temperature of -20°C until it was ready for use.

Molecular detection of E. histolytica

Following the microscopic examination, the identification of E. histolytica in stool samples was further confirmed through molecular analysis using PCR amplification of the SSU rDNA locus. For this purpose, 0.2 μM of both forward (EH1) and reverse (EH2) primers (**Table 3.1**), obtained from Eurofins, were utilized (Mukherjee et al., 2009). The PCR amplification was carried out in a 50 μl reaction mixture containing 2.5 U of TaKaRa r-Taq polymerase from Takara. The PCR amplification mixture included 3 μl of stool DNA. The PCR temperature cycling conditions were as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 51° C for 30 seconds, and polymerization at 72°C for 25 seconds. Finally, a final extension step was performed at 72°C for 7 minutes. The amplified PCR products were then separated by 1.5% agarose gel electrophoresis and visualized using a UV-transilluminator.

Molecular detection of G. lamblia

To confirm the presence of *G. lamblia* in clinical samples, a conventional PCR method was employed, targeting a 218-bp region of beta giardin (bg) gene of the parasite. The PCR amplification was carried out in a 50 μl reaction mixture using TaKaRa r-Taq polymerase from Takara. For the reaction, 2.5 U of TaKaRa Taq polymerase, along with nuclease-free water, 1X buffer with 1.5 mM MgCl2, and 200 μM of dNTPs (all from Takara), were utilized. For the PCR amplification, 0.2 μM of each forward primer (Gldt218F) and reverse primer (Gldt218R) from Eurofins were used (**Table 3.1**). The PCR reaction mixture included 2.5 U of TaKaRa Taq polymerase (from TaKaRa), along with nuclease-free water. A total of 3 μl of stool DNA was added to the reaction mixture. The PCR conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 64° C for 30 seconds, and extension at 72°C for 30 seconds. Finally, a final extension step was performed at 72°C for 7 minutes, and the reaction was then held at

4°C. The amplified PCR products were subjected to separation using 1.5% agarose gel electrophoresis and subsequently visualized using a UV-transilluminator.

Molecular detection of Cryptosporidium spp.

After the initial screening for *Cryptosporidium* spp. using microscopy, positive samples were further confirmed using a conventional PCR method targeting the 18S rRNA gene. For the PCR reaction, 2.5 U of TaKaRa Taq polymerase (from TaKaRa) was used, along with nuclease-free water and 1X buffer containing 1.5 mM MgCl2 (from TaKaRa). Additionally, 200 µM of dNTPs (from TaKaRa) were included. The PCR amplification employed 0.2 µM of each forward primer (Cryp18S_F) and reverse primer (Cryp18S_R) from Eurofins. The primer pair utilized in this study was originally adopted from the work of Xiao et al. in 1999 (Xiao et al., 1999) (**Table 3.1**). The PCR amplification mixture included 3 µl of template DNA. A total of 35 cycles were performed, with each cycle consisting of denaturation at 94°C for 35 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. The PCR reaction began with an initial hot start at 94°C for 5 minutes and concluded with a final extension step at 72°C for 7 minutes.

3.2.4. Data collection of other Enteropathogen infections:

The presence of helminth parasites was determined through the use of light microscopy and wet mount techniques. In addition to that, information regarding co-infections with other enteric pathogens such as *Vibrio cholera* O1/O139, *Salmonella* spp., *Campylobacter jejuni*, Rotavirus, astrovirus, *E. coli* and adenovirus was collected from the institutional database of ICMR-National Institute of Cholera and Enteric Diseases in Kolkata, India.

3.2.5. Species differentiation in *Cryptosporidium* infections

Distinguishing between various species of *Cryptosporidium* based on their oocysts is challenging as they appear similar. The five major species causing cryptosporidiosis are *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium felis*, and *Cryptosporidium canis*. Among these, *Cryptosporidium hominis* and *Cryptosporidium parvum* are the major causative agent for human cryptosporidiosis. The primer pair employed for molecular detection of *Cryptosporidium* spp. during our surveillance lacks the capability to distinguish between these different species. Therefore, in order to prevent and control cryptosporidiosis effectively, it is crucial to identify and characterize

different *Cryptosporidium* species, as well as determine their population variants such as genotypes and subtypes. These aspects play a fundamental role in studying the epidemiology of cryptosporidiosis.

In this study, the characterization of *Cryptosporidium* spp. was conducted using a PCR amplification technique targeting the 70 kDa Heat Shock Protein (HSP70). Subsequently, a 324 bp segment of this gene was subjected to bi-directional sequencing. A total of 28 samples tested positive for *Cryptosporidium* spp. were included in the study. These *Cryptosporidium* positive samples were obtained from the surveillance study mentioned earlier. We amplified the target locus using genus-specific primer pairs, CR_HSP70_FP and CR_HSP70_RP (**Table 3.1**). The PCR was carried out in a 50 µl reaction volume using 1X buffer containing 1.5 mM MgCl2 (Roche, Mannheim, Germany), 200 µM of each dNTP, 10 pmol of each primer (GCC Biotech, Joychandipur, India), 1 unit of Taq DNA polymerase enzyme (Roche), and 125 ng of isolated DNA. The PCR cycle conditions for gene amplification were as follows: an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes, and then the reaction was held at 4°C. To validate the results, both positive controls (containing *Cryptosporidium* DNA) and negative/no template controls (containing all PCR reagents without DNA template) were included in each round of PCR. After the PCR was completed, the PCR products were separated by electrophoresis for 35 minutes at 90 V in a 1.5% agarose gel to observe specific bands in the presence of a marker. Amplicons representing specific band sizes were extracted from the gels and purified using the Agarose Gel DNA Extraction Kit (Roche) following the manufacturer's protocol. The yield of the purified products was verified again by electrophoresis.

The purified products were then subjected to bidirectional automated sequencing using the Applied Biosystems BigDye Terminator V3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the same primers used for PCR amplification. The sequencing was performed using the ABI PRISMR3100 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). To determine the *Cryptosporidium* species/genotypes, the obtained sequences were compared with sequences in the GenBank database using a BLAST search. The levels of identity were established based on the comparison. The sequences were aligned using MultAlin to analyze the genetic variations and identify the specific *Cryptosporidium* species/genotypes present in the samples.

Table 3.1 Primer Sequences and Melting Temperatures (Tm) Used in Molecular Detection Methods.

Organism name	Target gene	Primer name	Primer sequence (5' to 3')	Annealing temp.	Product size
E. histolytica	18S rRNA	EH1	GTACAAAATGGCCAATTCATTCAATG	51° C	135 bp
		EH2	ACTACCAACTGATTGATAGATCAG		
G. lamblia	Beta-giardin	MAH433F	CATAACGACGCCATCGCGGCTCTCAGGAA	64° C	218 bp
		MAH592R	TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA		
Cryptosporidium spp. (for surveillance study)	18S rRNA	Cr18S_F2[a]	TAGGAGTAGGAGCTTCACC	55°C	826 - 864 bp
		Cr18S_R2[a]	AAGGAGTAAGGAACAACCTCCA		
Cryptosporidium spp. (for species differentiation)	HSP70	HSP70_FP	GCTGGTGATACTCACTTGGG	55 C	324bp
		HSP70_RP	TCTCTTGTCCATACCAGCA		

3.2.6. Evolutionary analysis of *Cryptosporidium* isolates

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura Nei 1993). The tree with the highest log likelihood (-548.58) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (8 categories (+*G*, parameter = 6.5241)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 285 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

3.3. Results

In this surveillance study, a comprehensive screening of samples was conducted, comprising 3258 samples from ID Hospital and 2793 samples from B C Roy Hospital. The gender distribution among the admitted patients revealed a male-to-female ratio of 1.24, indicating a slightly higher proportion of males in the study population.

3.3.1. Prevalence of *E. histolytica*

Conventional microscopy in saline wet mounts and Lugol's iodine wet mounts staining was employed to preliminary examine the presence of *Entamoeba* spp. Distinguishing *E. histolytica* from morphologically similar cysts and trophozoites of commensal *E. dispar* and amphizoic *E. moshkovskii* poses a challenge. Even distinguishing *E. hartmanni* and *E. bangladeshi* from *E. histolytica* is also difficult. As a result, we reported any species of *Entamoeba* based on microscopy after wet mount and iodine staining. Following light microscopy, we observed that 4.10% (n/N = 248/6051, 95% CI 0.036–0.046) of the samples showed positive cysts/trophozoites of *Entamoeba* spp. The microscopic images of various *Entamoeba* species are depicted in **Fig 3.1.**

A species-specific PCR assay (refer **Fig 3.2** for gel images) was conducted on all 6051 samples to identify the presence of *E. histolytica*. The results showed that 52 out of 6051

samples tested positive for *E. histolytica*, indicating a prevalence rate of 0.86% (n/N=52/6051, 95% CI 0.0065 to 0.0113). Out of the 52 samples that tested positive for *E. histolytica* through PCR, 45 exhibited cysts/trophozoites when examined under a light microscope, while 7 samples did not show any observable presence of cysts/trophozoites. Furthermore, the specific species of *Entamoeba* could not be determined in a total of 203 samples (n/N=203/248, 81.85%, 95% CI 0.7656 to 0.8618) during microscopy. This underscores the importance of conducting further molecular characterization of cases that tested positive under microscopy but were negative for *E. histolytica* in PCR. This will help identify the other *Entamoeba* species involved in diarrheal incidence in this area. These results also indicate that PCR is a preferable method for accurate diagnosis of amoebiasis.

Trichrome staining was also performed for the 45 samples, which were positive for *E. histolytica* in saline wet mounts/Lugol's iodine wet mounts and PCR. After trichrome staining we observed that 53.33% (n/N=24/45, 95% CI 0.3908 to 0.6707) contained hematophagous trophozoites, indicating the presence of colon-invasive forms of *E. histolytica*. Additionally, 20% (n/N=9/45, 95% CI 0.1067 to 0.3404) of the positive samples contained non-hematophagous trophozoites, which are typically found in the lumen of *E. histolytica*. In the remaining 26.67% (n/N=12/45, 95% CI 0.1584 to 0.4116) of *E. histolytica* positive samples, no trophozoites (hematophagous or non-hematophagous) were detected; only cysts were observed.

The gender distribution in *E. histolytica* infection revealed that it was more prevalent in males (n/N= 38/3354, p=0.0112) compared to females (n/N= 14/2697). Among the age groups, the highest infection rate of *E. histolytica* was observed in the 30-50 years age group (0.71%, 95% CI 0.64- 4.10), while the lowest infection rate was found in the 0-4 years age group (1.25%, 95% CI 0.36-2.25). However, the occurrence of infections was not statistically associated with age groups **(Table 3.3)**.

Table 3.2 Multinomial Logistic Regression Models examining gender and age groups as significant risk factors for *E. histolytica* infection (*statistical significance assessed via Chi-square test with $p < 0.05$).

Category	**Group**	**% of positive case**	**OR**	**95% CI**	**P value**
Gender	Male	1.13%	2.20	1.19- 4.01	*0.0112
	Female	0.52%	-	-	-
Age	0-4 yrs	0.71%	0.9	0.36-2.25	0.81
	5-12 yrs	0.84%	1.08	0.38-3.63	>0.999
	13-18 yrs	0.78%	1.00	-	-
	19-29 yrs	0.77%	0.98	0.34-3.30	>0.999
	30-50 yrs	1.25%	1.60	0.64- 4.10	0.48
	>50	0.90%	1.57	0.54-4.50	0.43

*value analyzed by Chi-square test is considered statically significant when $p < 0.05$

3.3.2. Prevalence of *G. lamblia*

The prevalence of *G. lamblia*, as determined by conventional microscopy (**Fig 3.1**) in this study, was found to be 6.18% (n/N=374/6051; 95% CI: 0.0560 to 0.0682). However, when PCR screening was employed (refer **Fig 3.2** for gel images), a total of 398 positive samples were identified, including those that were detected through microscopy. This resulted in an overall prevalence of 6.57% (n/N=398/6051, 95% CI 0.0598 to 0.0723). The analysis further revealed that infections caused by *G. lamblia* were more commonly observed among children aged between 0 and 4 years old (OR: 2.26, 95% CI: 1.58-3.21, p <0.0001). These findings indicate that children under the age of 4 face a significantly higher risk of contracting a *Giardia* infection. Statistical data indicates that the age groups of 5-12 years and 13-18 years are also considered to be at risk, although their risk is comparatively lower than the age group under 4 years. The gender distribution of *G. lamblia* infection indicated a slightly higher prevalence in males compared to females, but the difference was not statistically significant (OR: 1.044, 95% CI: 0.84- 1.29, p=0.69) (**Table 3.3)**.

Table 3.3 Multinomial Logistic Regression Models examining gender and age groups as significant risk factors for *G. lamblia* infection (*statistical significance assessed via Fisher's exact test with $p < 0.05$).

Category	Group	% of positive case	OR	95% CI	P value
	Male	6.29%	1.044	0.84- 1.29	0.69
Gender	Female	6.04%	-	-	-
	0-4 yrs	8.06%	2.26	1.58-3.21	<0.0001*
	5-12 yrs	10.30%	2.96	1.96-4.45	<0.0001*
Age	13-18 yrs	7.18%	1.99	1.31-3.00	0.011*
	19-29 yrs	3.22%	0.86	0.50- 1.47	0.68
	30-50 yrs	3.74%	1	-	-
	>50	3.15%	0.84	0.50-1.38	0.48

*value analyzed by Fisher's exact test is considered statically significant when $p < 0.05$

3.3.3. Prevalence of *Cryptosporidium* spp.

Out of 6051 diarrhoeal samples screened, 4.36% (n/N= 264/6051; 95% CI 0.0388 to 0.0491) of the samples tested positive for *Cryptosporidium* spp. using Kinyoun's staining **(Fig 3.1)**. However, when PCR screening (refer **Fig 3.2** for gel images) was utilized, a total of 283 positive samples were identified, including those previously detected through microscopy. As a result, the overall prevalence of *Cryptosporidium* infection was found to be 4.68% (n/N= 283/6051, 95% CI 0.0417 to 0.0524). When analyzing various age groups, it was found that the highest incidence of cryptosporidiosis was observed among children aged 0 to 4 years (OR: 1.55, 95% CI 1.09-2.21, p= 0.017), surpassing the prevalence rates in other age groups. However, the distribution of *Cryptosporidium* spp. did not show a significant association with gender (p=0.71) (**Table 3.4)**.

Table 3.4 Multinomial Logistic Regression Models examining gender and age groups as significant risk factors for *Cryptosporidium* spp. infection (*statistical significance assessed via Fisher's exact test with p < 0.05).

Category	Group	% of positive case	OR	95% CI	P value
Gender	Male	4.71%	1.052	0.83-1.34	0.71
	Female	4.49%	-	-	-
Age	0-4 yrs	5.98%	1.55	1.09-2.21	0.017*
	5-12 yrs	4.39%	1.12	0.69-1.87	0.70
	13-18 yrs	3.26%	0.83	0.5-1.4	0.53
	19-29 yrs	3.52%	0.89	0.54- 1.45	0.7
	30-50 yrs	3.92%	1.0	-	-
	>50	4.72%	1.23	0.79-1.88	0.43

*value analyzed by Fisher's exact test is considered statically significant when p < 0.05

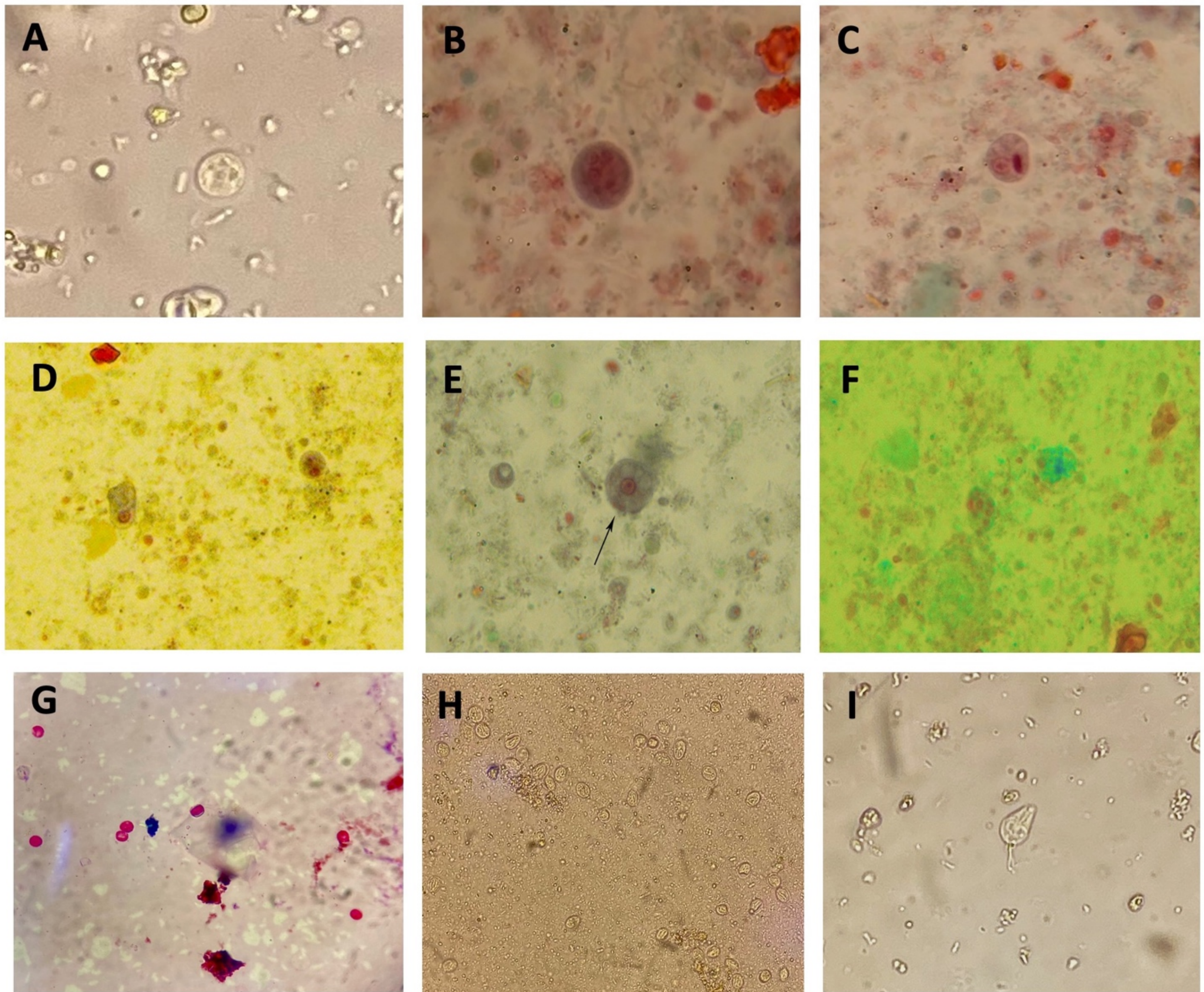


Fig. 3.1 Microscopic view of different common enteric parasites. **A.** *Entamoeba* cyst after wet mount. **B** and **C.** *Entamoeba* cyst after iodine staining. **D** and **E.** *Entamoeba* trophozoites after Trichrome staining. **F.** Hematophagous Trophozoites of *E. histolytica*. **G.** *Cryptosporidium* spp after acid fast staining. **H** and **I.** Cyst and Trophozoites of *Giardia lamblia* respectively.

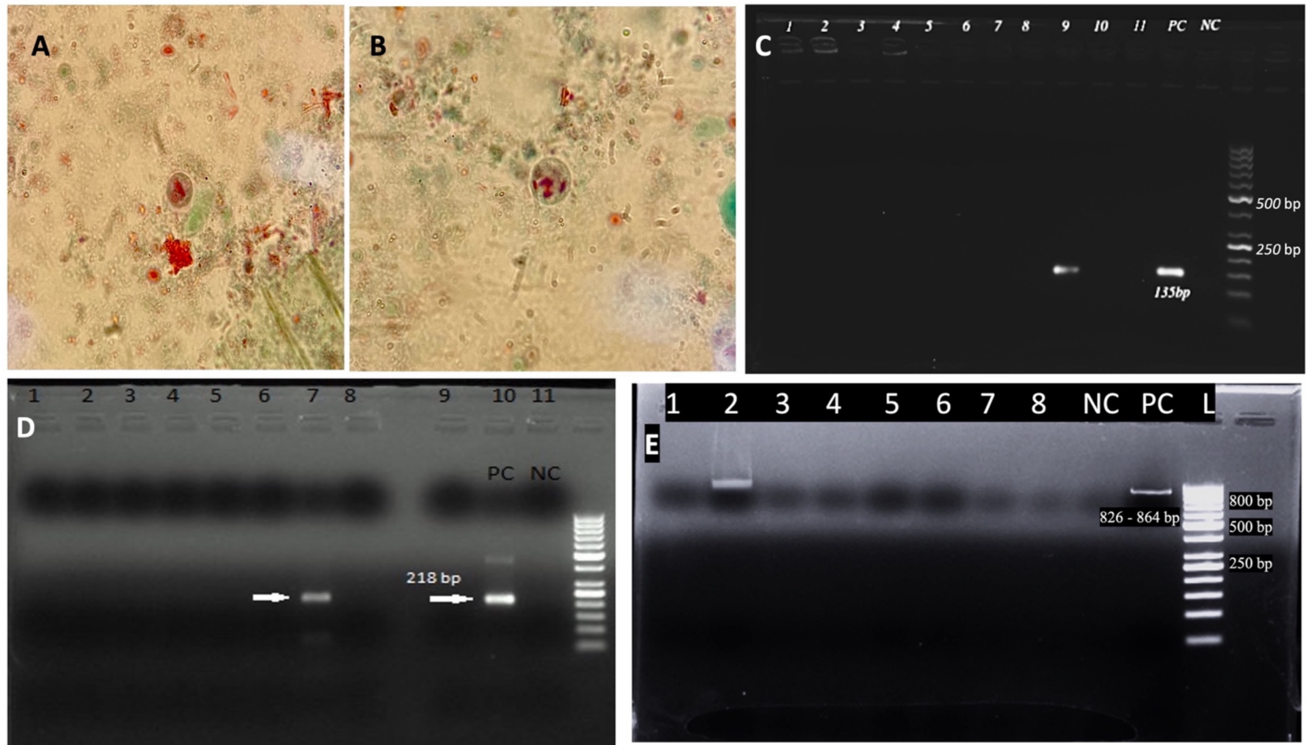


Fig 3.2 A and **B.** Microscopic examination of *Entamoeba* cysts. **C.** Gel electrophoresis demonstrating the detection of *E. histolytica*. **D.** Gel electrophoresis demonstrating the detection of *G. lamblia*. **E.** Gel electrophoresis demonstrating the detection of *Cryptosporidium*.

3.3.4. Additional enteropathogens in parasite-infected diarrhoeal patients

Out of the total 52 positive cases of *E. histolytica*, 61.54% (n/N=32/52, 95% CI 0.4794 to 0.7355) were solely infected with *E. histolytica*, while the remaining cases had combined infections involving other enteric parasites, viruses, or bacteria. Upon microscopic examination, we identified hematophagous trophozoites in 24 cases. Intriguingly, 66.66% (n/N= 16/24, 95% CI 0.4658 to 0.8216) of the cases with sole *E. histolytica* infections exhibited colon-invasive forms, as evidenced by the presence of hematophagous trophozoites in the microscopy samples. Among the cases with non-hematophagous trophozoites of *E. histolytica*, 77.77% (n/N=7/9, 95% CI 0.4428 to 0.9466) were solely infected with *E. histolytica*, while the remaining 22.22% (n/N=2/9, 95% CI 0.0534 to 0.5572) had coinfections involving diarrheagenic bacterial infections.

Out of a total of 398 cases of *Giardia lamblia* infection, 44.22% (n/N=176/398, 95% CI 0.3942 to 0.4913) were identified as sole infections, while the remaining cases (55.78%) were characterized by co-infections with other commonly found enteric pathogens. It is worth mentioning that mixed infections with *Giardia* were primarily associated with *Vibrio cholerae*, Rotavirus, and *E. coli*.

In the case of *Cryptosporidium* infection, 53.40% (n/N= 141/264, 95% CI 0.4739 to 0.5933) cases were solely infected with *Cryptosporidium* spp. The remaining 46.60% (n/N= 123/264, 0.4067 to 0.5261) were coinfected with other diarrheagenic parasites, bacteria or viruses. *Cryptosporidium* infection showed a higher tendency to be coinfected with viruses than other bacteria or parasites. 71.15% of coinfected cases were involved with diarrheagenic viral infections for instance Adenovirus, rotavirus or astrovirus. The coinfection incidence of diarrheagenic virus and *Cryptosporidium* spp. can be the outcome of weakened immune systems of the individuals.

3.3.5. Seasonal pattern of diarrheagenic enteric parasite infection

Kolkata is characterized by a tropical climate, which manifests in the presence of five distinct seasons throughout the year. These seasons are categorized as summer (April to May), monsoon (June to September), post-monsoon/Autumn (October to November), winter (December to January), and pre-monsoon/Spring (February to March) (Chowdhuri et al. 2022).

In this hospital-based survey, we found that *G. lamblia* remains a prominent pathogen frequently detected in diarrheal samples. Our study consistently revealed a high infection rate

of *Giardia* throughout the observation period. Data collected over a span of three years (2017 to February 2020) consistently revealed a significant increase in *Giardia* infection cases from the spring season to the post-monsoon season. Conversely, a decline in *Giardia* infection was observed during the winter season **(Fig. 3.3)**.

Although sporadic infections of *E. histolytica* are observed throughout the year, there is a noticeable increase in *E. histolytica* infection rates during the post-monsoon season. However, due to the overall low prevalence of *E. histolytica* infections (<1%), it is challenging to draw specific trends or conclusive findings. The presence of higher standard deviations (SD) in the graph within each season suggests the absence of consistent seasonal trends in *E. histolytica* infections **(Fig. 3.3)**.

A notable pattern of increased incidence of *Cryptosporidium* infection has been noted during post-winter (Spring) and post-monsoon seasons (August to October) **(Fig. 3.3)**. This trend aligns with findings reported by Mukherjee et al. in 2009, who conducted a hospital-based surveillance study in the vicinity of Kolkata.

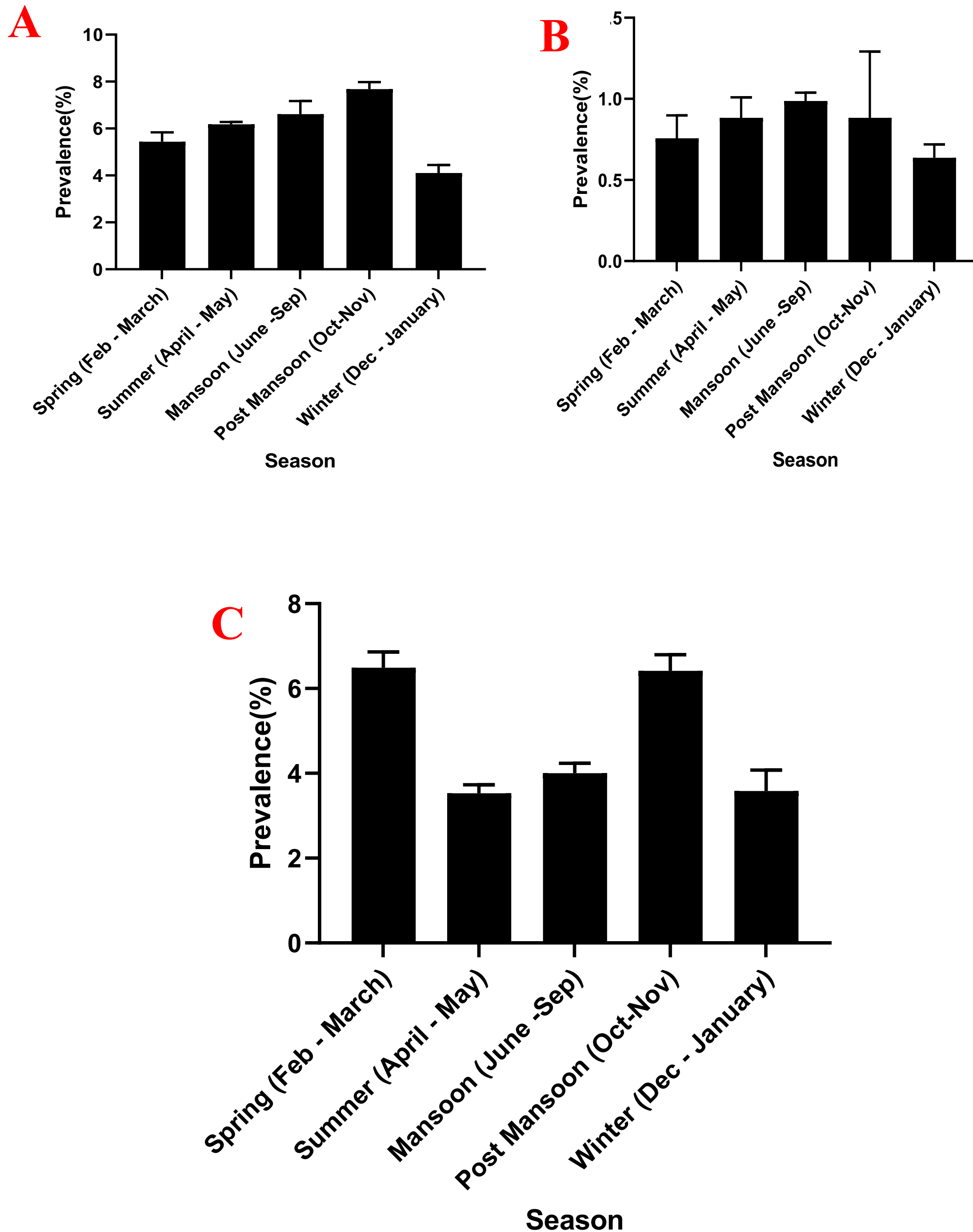


Fig. 3.3 Seasonal distribution of parasitic infections in Kolkata and its surrounding areas in three consecutive years (2017 to February 2020). A. Seasonal Distribution of *Giardia lamblia* Infection: This graph illustrates the variation in the occurrence of *Giardia lamblia* infections throughout the year in Kolkata and its surrounding regions. The data reveals the seasonal patterns of *G. lamblia* infection. **B.** Seasonal Distribution of *Entamoeba histolytica* Infection: The graph displays the seasonal trends of *Entamoeba histolytica* infections in Kolkata and its neighbouring areas. **C.** Seasonal Distribution of *Cryptosporidium* spp. Infection: This graph showcases the seasonal distribution of *Cryptosporidium* spp. infections in Kolkata and the surrounding regions.

3.3.6. Species distribution of *Cryptosporidium* spp.

A total of 28 *Cryptosporidium* samples were analysed, and the results showed that 53.57% (n/N=15/28, 95% CI 0.3581 to 0.7047) of the samples were identified as *C. hominis*, while 39.29% (n/N=11/28, 95% CI 0.2352 to 0.5763) were identified as *C. parvum*. Interestingly, 7.14% of the samples belonged to *C. viatorum* (refer **Fig. 3.4** for microscopic view), which is a species that was recently described in 2012. Notably, there have been no reports of *C. viatorum* incidence in eastern India (refer to **Fig. 3.5** for PCR amplification). The sequences obtained from *C. hominis* and *C. parvum* have been submitted to NCBI GenBank and can be accessed using the accession numbers OR133932-OR133946 and OR134008- OR134018, respectively. Similarly, the sequences of *C. viatorum* have been submitted under the accession number MT341769.1 and OP420532.1 (refer to **Fig. 3.6** for multiple alignment of the sequences with other reference sequences).

We found that the two isolates of *C. viatorum* showed complete similarity to three recently identified *C. viatorum* isolates (GenBank Accession Nos. JX978274.1, JX978273.1, and JN846706.1). In the case of *C. hominis*, we identified four distinct haplotypes, which we designated as Ch_Hap1, Ch_Hap2, Ch_Hap3 and Ch_Hap4. Ch_Hap1 was identical to the reference strains of *C. hominis* (GenBank Accession No. KX926461.1 and CryptoDB: CHUDEA2_20). The remaining isolates of *C. hominis* showed variations from Ch_Hap1 by one to four single-nucleotide polymorphisms (SNPs). In Ch_Hap2, a single SNP was observed at position 699, where T was replaced by G (699T/G). Ch_Hap3 exhibited SNPs at positions 728T/C, 773C/G, 775T/G, and 776C/A. Ch_Hap4 displayed SNPs at positions 728T/C, 773C/G, 775T/G, 776C/A, and 977A/C. The analysis of the genetic diversity indices in the C. hominis population revealed the presence of 6 polymorphic sites, all of which were informative for parsimony analysis. No singleton-variable sites were observed. This indicates that there are multiple variations in the genetic makeup of this population. Additionally, the haplotype diversity (HD) was calculated to be 0.771±0.058, suggesting a high level of diversity among the different haplotypes present. The nucleotide diversity (π) was found to be 0.01±0.001, indicating a higher genetic variation at the nucleotide level (**Table 3.5**).

These findings suggest that the *C. hominis* population in this specific geographical area exhibits a high level of genetic diversity. The presence of informative polymorphic sites, along with a notable recombination rate, further supports the notion of genetic variation within

the population. This information provides insights into the evolutionary dynamics and genetic structure of *C. hominis* in this particular region.

Within the *C. parvum* population, we have observed the presence of two distinct haplotypes known as Cp_Hap1 and Cp_Hap2. Cp_Hap1 exhibited an identical genetic makeup to the reference strains of *C. parvum*, as indicated by the matching sequences of GenBank Accession No. MK609845.1 and CryptoDB: cgd2_20. However, Cp_Hap2 displayed slight genetic variations compared to Cp_Hap1, specifically at positions 900C/T, 901A/C, and 948A/G, where one to three single-nucleotide polymorphisms (SNPs) were detected. During the study of genetic diversity indices in the *C. parvum* population, three polymorphic sites were identified, all of which were informative for parsimony analysis. Interestingly, no singleton-variable sites were detected. Notably, this population displayed a relatively lower Hd value of 0.327±0.153 and a nucleotide diversity (π) of 0.00344±0.00161. These findings indicate a lower level of genetic variation within the *C. parvum* population compared to the *C. hominis* population.

Figure 3.7 presents a constructed phylogenetic tree that demonstrates the clear separation of three species lineages. These lineages form distinct clades, and there is a notable presence of genetic variation, or polymorphism, observed within the partial fragments of the gene. Importantly, the isolates obtained in our study were found to cluster together with reference species strains that have been previously described. The strength of these clades is reinforced by the high bootstrap values, indicating robust support for their grouping. The newly discovered isolates belonging to the identified species were successfully classified within their respective species group and can be designated as new variants. This *C. viatorum* clade was the most outlying group with a robust bootstrap value of 100%, distinguishing it from the other two species.

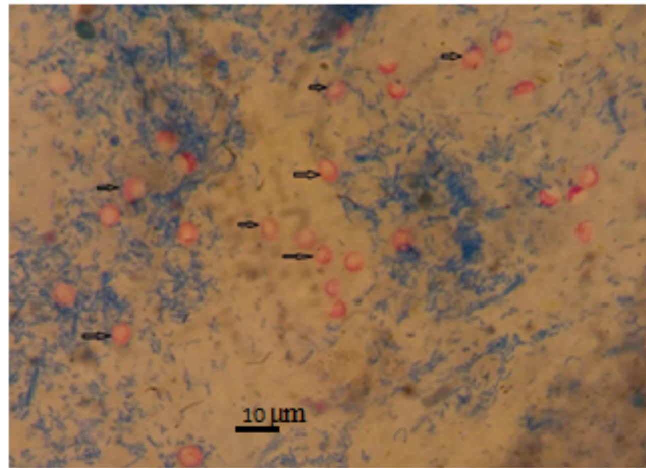


Fig. 3.4 Microscopic view of *C. viatorum* Oocytes after modified acid-fast staining. (100X).

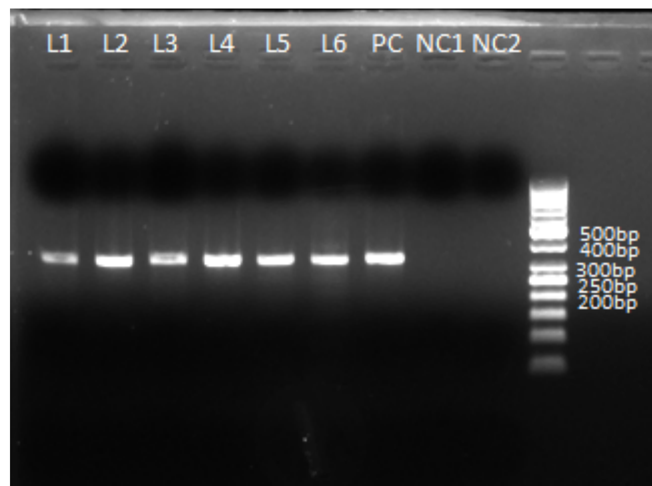


Fig. 3.5 Amplification of *HSP70* gene of *Cryptosporidium* spp. of 6 microscopy positive samples. PCR product size: 324bpp L1-L6 are amplified PCR products of *Cryptosporidium* spp. PC is amplified PCR product of *Cryptosporidium parvum* as a positive control. NC1 and NC2 are negative control contain no stool DNA.

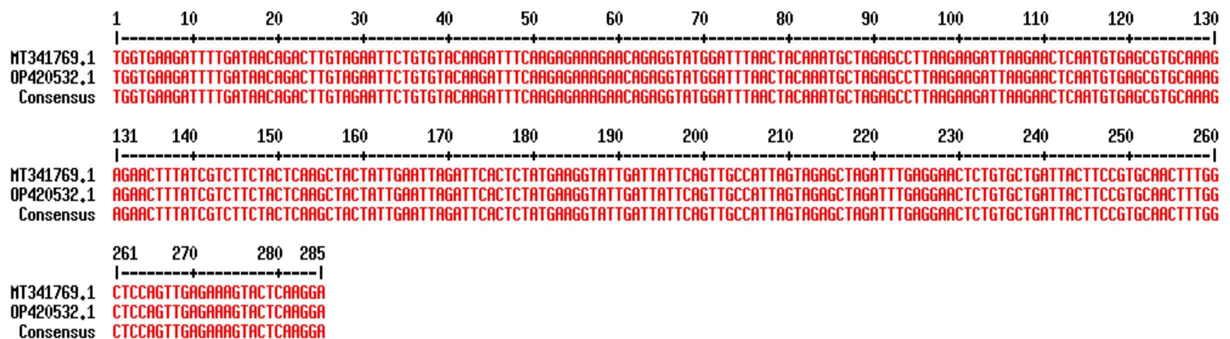


Fig 3.6 Alignment of 2 isolates (GenBank MT341769.1 and OP420532.1) of *C. viatoram* and reference strain using ***MultAlin*** after sequencing; the alignment shows 100% similarity with the reference strain.

Table 3.5 Summary table of Haplotype diversity of *Cryptosporidium* spp. population (N= Number of sequences of isolates, S= Number of variable sites, H= Haplotypes, Hd = Haplotype diversity, Π = Nucleotide diversity, K = average number of nucleotide differences)

Population	N	S	H	Hd ±S.D.	Π ±S.D.	K
C. hominis	15	6	4	0.771±0.058	0.00344±0.00161	2.72
C. parvum	11	3	2	0.327±0.153	0.00344±0.00161	0.98

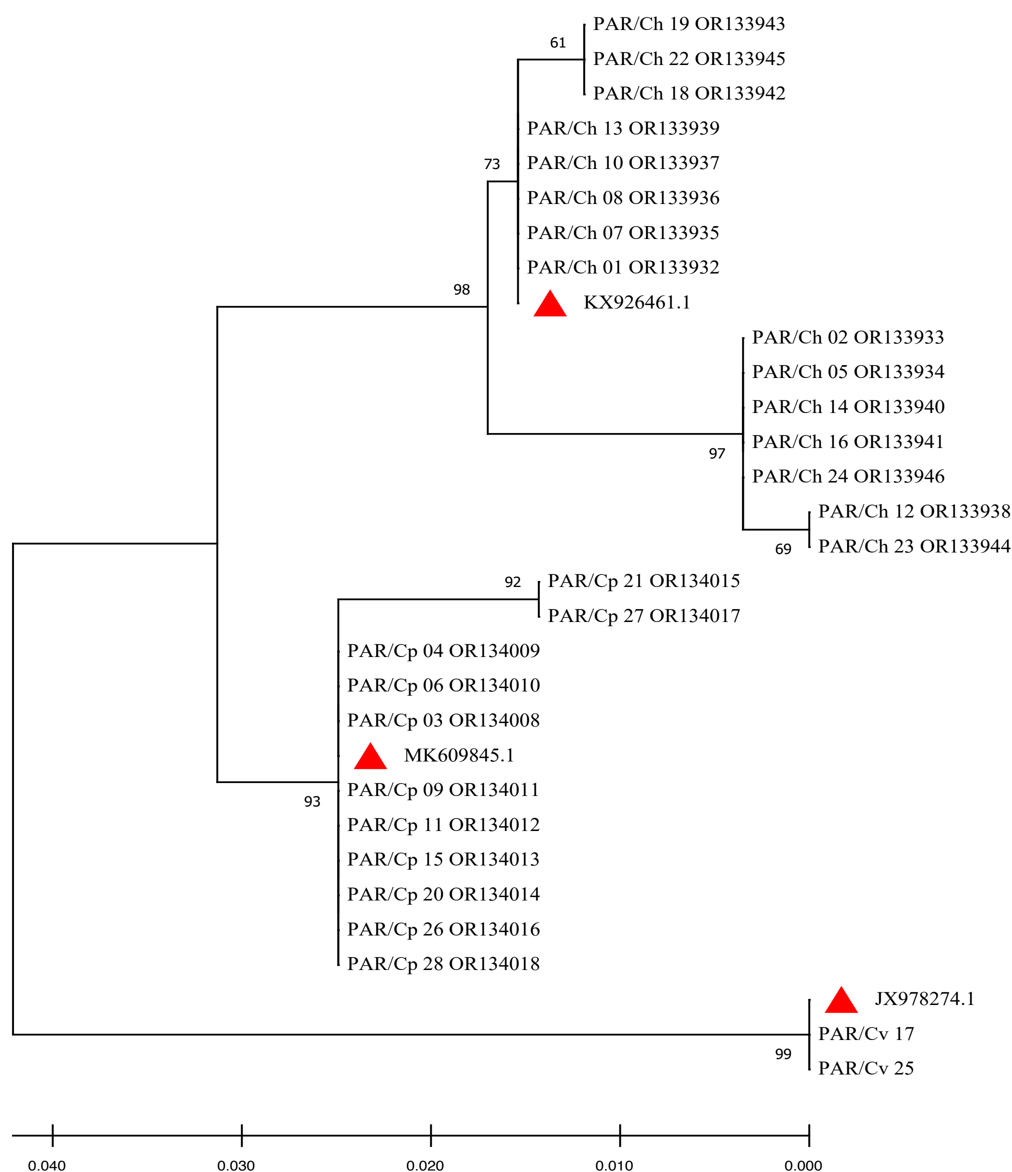


Fig. 3.7. Phylogenetic analysis of *Cryptosporidium* spp isolates obtained in Kolkata and adjacent areas.

3.4. Discussion

In developing countries, infectious intestinal diseases are frequently managed without undergoing thorough diagnostic procedures or obtaining information regarding their underlying causes, as they are often self-limiting in nature. From a public health perspective, having information about the specific pathogenic organism responsible for infectious intestinal diseases is crucial for appropriate treatment, monitoring trends, serving as an early warning system to identify outbreaks, and implementing control measures and health policies. In this study, we conducted a comprehensive assessment of the current prevalence of *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp in Kolkata and its surrounding areas. The detection methodology involved meticulous microscopic observation of slides, followed by PCR analysis. PCR is strongly recommended, especially when dealing with samples that initially exhibit a low cyst load. Furthermore, in cases requiring accurate diagnosis, PCR becomes a necessary technique due to the presence of numerous morphologically identical species, which demand specialized expertise, particularly in instances of *Entamoeba* and *Cryptosporidium* infections. In our study, we also observed that PCR significantly improves the sensitivity of diagnosing enteric parasitic infections. Our molecular method based on PCR successfully detects the presence of parasite DNA in samples that were previously identified as negative through microscopic examination. As a result, for achieving accurate positive results, it is strongly recommended to employ both microscopic examination and PCR simultaneously. (Sari et al. 2019).

This study documented a consistent infection rate of *Giardia* without any noticeable seasonal variations. In this region, we observed a substantial decrease in the prevalence of giardiasis or *Giardia* induced diarrhoea, with a reduction from 13% to 6.18% compared to the previous decade. (Mukherjee et al. 2009). The observed decline in prevalence can be partially attributed to the implementation of improved water supply and sanitation measures by the government of India. These efforts may have played a role in the partial eradication of giardiasis. Despite continuous efforts, the prevalence of giardiasis among children below 12 years of age remains alarmingly high. Particularly, individuals residing in economically disadvantaged areas, including both rural regions and suburbs of Kolkata, exhibit the highest infection rates. In low- and middle-income countries, giardiasis has been linked to impaired growth and diminished cognitive function, especially in malnourished children under the age of 5 (Shaima et al. 2021). A study conducted in Mexico has established a correlation between giardiasis and vitamin A deficiency in schoolchildren (Quihui-Cota et al. 2008). These findings highlight the adverse

effects of chronic *Giardia* infection on the physical and mental development of children, ultimately impacting human resources and the economy of a country as a whole. The study conducted in the specified setting revealed a notable prevalence of co-infection cases in *Giardia* infection. This observation indicates that *G. lamblia* has the capacity to efficiently parasitize the intestinal tract of the host without causing any symptoms. In this area, a lower prevalence of sole *Giardia* infections was observed, while a higher occurrence of co-infection cases with other enteric pathogens was found. Numerous epidemiological studies conducted in endemic regions worldwide have consistently reported a lack of positive correlation between *Giardia* infections and the presence of diarrhoea. These findings emphasize that asymptomatic giardiasis is prevalent in certain environments, representing the usual scenario rather than the exception (Bartelt et al. 2016; Koster et al. 2021).

We have also conducted an evaluation of *Cryptosporidium* infections among individuals with diarrhoea. Interestingly, the occurrence of *Cryptosporidium* infection displayed distinct seasonal patterns when compared to other parasitic infections. We observed two notable peaks during the spring season and after the monsoon period. The findings of Das et al. in 2006 also reported similar results; however, their study focused exclusively on children between the ages of 0 and 5 years (Das et al. 2006). Another noteworthy aspect of our surveillance study is the frequent occurrence of coinfection between *Cryptosporidium* infection and diarrhoeagenic virus infection. A study conducted by Naif et al. in 2020 revealed that Astrovirus and *Cryptosporidium* frequently occur as co-infections (Naif et al. 2020). Additionally, *Cryptosporidium* spp. is a major opportunistic infection associated with HIV (Ahmadpour et al. 2020). However, there is a notable research gap when it comes to assessing the trend of co-infection between diarrhoeagenic viruses and *Cryptosporidium* spp. It is possible that the weakened immune systems observed in children and the elderly may contribute to the incidence of such co-infections. Our study was conducted at two government hospitals that predominantly serve patients from lower socioeconomic backgrounds. Due to the lower socioeconomic status of these individuals, many of them suffer from poor nutrition, which can compromise their immune response (Maggini et al. 2018). The weakened immune system in the elderly population can also be attributed to factors such as smoking and alcohol consumption, as supported by previous research (Sarkar et al. 2015; Nouri-Shirazi and Guinet 2003). This compromised immune response could potentially worsen the incidence and severity of both *Cryptosporidium* and diarrhoeagenic viral infections. However, in order to

draw definitive conclusions, a comprehensive epidemiological study should be conducted to investigate this relationship in detail.

The tendency for coinfection can also be influenced by the intricate ecosystem of gut microbes. The intestinal immune system has evolved to effectively respond to the presence of diverse microorganisms, encompassing beneficial, harmful, and neutral microbes (Hooper et al. 2012). The intestinal immune system of the host must accurately evaluate whether to accept or reject new microbes in different situations. At the same time, the incoming organisms have also evolved to optimize their chances of being accepted (Reynolds et al. 2015). Infections caused by diarrheagenic viruses like rotavirus can cause temporary changes in the gut microbiota composition. The microenvironment of the gut may contribute to the determination of the severity of a *Cryptosporidium* infection (Carey et al. 2021). Furthermore, *Cryptosporidium* species are opportunistic apicomplexan parasites that specifically infect the epithelial cells of the gut, resulting in debilitating gastrointestinal symptoms and diarrhoea in the host. This condition can create a favourable environment for the proliferation of other diarrheagenic pathogens, including viruses (Charania et al. 2020).

Molecular epidemiological studies on cryptosporidiosis have contributed to our enhanced comprehension of the transmission of *Cryptosporidium* species and its importance in public health concerning humans, animals, and the environment (Areeshi et al. 2007). Through DNA sequencing of the positive samples, it was determined that *C. hominis* is the predominant species in this region, followed by *C. parvum*. Interestingly, no significant differences in clinical phenotypes were observed. In addition to these two prevalent *Cryptosporidium* species, we also identified cases of diarrheal incidence caused by a recently described species of infection, *C. viatorum*.

While the number of patients infected with *C. viatorum* may not be substantial, it should not be overlooked. The oocysts of *C. viatorum* exhibit morphology and staining characteristics typical of the genus, similar to *C. hominis* and *C. parvum*, which are the primary infective species in humans. This suggests that current microscopic studies are unable to differentiate *C. viatorum* from other *Cryptosporidium* species. Therefore, we strongly recommend employing molecular diagnostic techniques such as PCR and DNA sequencing for accurate identification of this species. Interestingly, we did not detect any single nucleotide polymorphisms (SNPs) in the examined locus for the *C. viatorum* isolates obtained in Kolkata.

Two cases of *C. hominis* infection were identified, both of which occurred in the age group of 5-12 years. The symptoms of *C. viatorum* infection, including diarrhoea, abdominal pain, fever, and nausea, closely resembled those seen in cases of *C. hominis* and *C. parvum* infections. However, unlike typical cryptosporidiosis cases, excessive vomiting was not reported in the individuals infected with *C. viatorum*.

C. viatorum was initially discovered in 2012 among travellers who had returned to Great Britain from the Indian subcontinent, experiencing gastrointestinal symptoms (Elwin et al. 2012). Natural infections with *C. viatorum* lead to symptoms such as diarrhoea, abdominal pain, fever, and occasionally nausea, which are characteristic of human cryptosporidiosis. These findings align well with our own results (Elwin et al. 2012). *C. viatorum* is an enteric parasite with a global distribution and has been identified in numerous patients who have travelled to Kenya, Guatemala, and the Indian subcontinent (Lebbad et al. 2013). To date, only one report of *C. viatorum* infection from India has been documented (Khalil et al. 2018). However, the significant prevalence of *C. viatorum* infection in Kolkata highlights the need for additional studies to investigate the phylogeography, host specificity, host-parasite interactions, and clinical significance of this relatively understudied parasite in the Indian population, as well as in animals. Further research is essential to gain a better understanding of *C. viatorum* in India and its potential implications.

In India, the potential for zoonotic transmission of various *Cryptosporidium* species between cattle and humans has been previously reported (Das et al. 2019). According to a study by Koehler et al. in 2018, Australian swamp rats have been identified as non-human hosts of *C. viatorum* (Koehler et al. 2018). This finding suggests that *C. viatorum* has the potential for zoonotic transmission. Recent studies suggest that *C. viatorum* may have originated as a species of rodents, indicating that rodents are likely to play a significant role as reservoir hosts (Koehler et al. 2018). Therefore, the infection reported in this study could potentially be a case of zoonotic transmission. However, it is important to highlight that as of now, no animal host for *C. viatorum* has been reported in India. Therefore, this study strongly suggests the need to identify the natural reservoir of *C. viatorum*. Such identification will greatly contribute to the effective control and management of this infection.

The other major enteric parasite that causes diarrhoea is *E. histolytica*. the microscopic detection of *E. histolytica* often leads to an overestimation of the number of infected individuals. Numerous studies have demonstrated that the coproscopic diagnosis of this intestinal protozoan is neither accurate in terms of specificity nor sensitivity (Ngui et al. 2012; Morsy et al. 2022). On the other hand, PCR-based assays not only prevent misdiagnosis but also reduce overtreatment (Leiva et al. 2006). When considering amoebic infection in the diarrheal patients studied, the prevalence rate of *E. histolytica* is lower compared to *Giardia* and *Cryptosporidium* infections. In 2009, Mukherjee et al. conducted a surveillance study in the same area, revealing an *E. histolytica* infection rate of 4.6% among the entire study population (Mukherjee et al. 2009). However, the current study indicates a decrease in *E. histolytica* prevalence to less than 1% in Kolkata and its surrounding areas. This reduction can possibly be attributed to the implementation of enhanced sanitation and improved water supply measures.

Accurately diagnosing *E. histolytica* infection is crucial for both individuals suffering from amoebic dysentery and those who are asymptomatic carriers. This is particularly significant in developing countries where poor hygienic conditions and insufficient water treatment facilities make person-to-person transmission highly likely (Servián et al. 2022). Through our microscopic screening, we identified a significant proportion of morphologically indistinguishable *Entamoeba* species in samples from individuals with diarrhoea. This underscores the importance of accurate diagnosis of amoebiasis and suggests that these *Entamoeba* species may have a potential pathogenic role in the occurrence of diarrheal cases. This study represents the initial evidence pointing towards a potential pathogenic role of previously considered non-pathogenic commensal *Entamoeba* species in eastern India. Presently, this genus comprises a minimum of seven species that can infect the human intestinal lumen, namely: *E. histolytica, E. dispar, E. moshkovskii, E. bangladeshi, E. coli, E. hartmanni,* and *E. polecki.* The cysts and trophozoites of the first four species are indistinguishable in terms of their morphology. Infections caused by *E. polecki* and *E. hartmanni*, which have cysts that can be mistakenly identified as immature forms of *E. histolytica*, are seldom associated with disease (Servián et al. 2022). Apart from the known species that infect humans, *E. nuttalli*, primarily found in nonhuman primates, was identified in a zoo caretaker (Servián et al. 2022). The less researched *Entamoeba* species found in the human intestine could potentially contribute to a significant portion of the microscopically unidentified *Entamoeba* species observed in our surveillance study. In the next chapter, we will disclose the distribution of these

unidentified *Entamoeba* species that are commonly found in diarrheal patients with other prevalent enteric parasites.

3.5. Conclusion:

In summary, the current study emphasizes that the occurrence of parasitic diarrhoea in humans remains a significant issue in the study area. The study underscores the necessity for additional research to comprehend the transmission patterns of the parasites and establish effective diagnostic measures to prevent the emergence of drug-resistant strains. This study also identified new eneteric parasite like *C. viatorum* those are not previously reported from this area, which is a major concern in aspect of parasitic infection.

Furthermore, it highlights the urgent need for public health authorities to implement prevention and control strategies to combat parasitic infections. These study findings emphasize the significance of accurate diagnosis and effective control measures in addressing the incidence of diarrhoea caused by parasites.

CHAPTER IV

INVESTIGATING THE PREVALENCE OF MORPHOLOGICALLY INDISTINGUISHABLE *ENTAMOEBA* SPECIES IN DIARRHEAL PATIENTS

Investigating the prevalence of morphologically indistinguishable *Entamoeba* species in diarrheal patients

4.1. Background

Infections caused by *Entamoeba* spp. are widespread. They are particularly prevalent in tropical countries that have lower socioeconomic communities and inadequate sanitation conditions. This alarming trend highlights the significant burden of these infections in such regions (Ngui et al. 2011). Traditionally, the diagnosis of an intestinal infection caused by *E. histolytica* involves examining fecal samples under a microscope. This method requires the microscopist to skilfully and patiently distinguish the cysts and trophozoites of *E. histolytica* from those of other non-pathogenic species that have distinct morphological features. Hence, identifying these cysts and trophozoites demands expertise and careful attention from the individual performing the microscopic examination.

Among the various species of *Entamoeba* that can be found in humans, one particular species, *Entamoeba histolytica*, has consistently been recognized as pathogenic since its discovery (Singh et al. 2021). However, ongoing debates have arisen due to occasional cases of infections caused by *E. dispar* and *E. moshkovskii*, leading to discussions about the classification of various Entamoeba species as either commensals or pathogens. This is because the genus includes multiple species that can reside in the human intestinal lumen and occasionally result in episodes of diarrhoea. These two species (*E. dispar* and *E. moshkovskii*) were previously regarded as commensals, but their pathogenic potential has been a subject of contention (Oliveira et al. 2015, Shimokawa et al. 2012).

E. histolytica is known to possess the capability to cause both invasive and non-invasive infections. On the other hand, *E. dispar* and *E. moshkovskii* are classified as species associated only with non-invasive infections (Uslu et al. 2016). The presence of red blood cells within trophozoites of *E. histolytica* facilitates the distinction between this species and *E. dispar* and *E. moshkovskii*. Recent research has brought attention to the existence of *E. moshkovskii*, another species of *Entamoeba* capable of causing infections in humans. Furthermore, the occurrence of *E. bangladeshi*, a pathogen responsible for causing diarrhoea in children, has been documented (Royer et al., 2012). However, it is important to note that the epidemiological

data pertaining to most resource-limited endemic countries heavily relies on microscopy, which exhibits significant limitations in terms of sensitivity (Singh et al. 2021). As a result, distinguishing between the morphologically identical *E. histolytica*, *E. dispar*, and E. moshkovskii is frequently challenging. (Gomes et al. 2014). A more accurate approach for reporting the microscopic findings would be to state "stool samples positive for *E. histolytica/dispar/moshkovskii/bangladeshi*" based on the initial recommendations provided by the World Health Organization (WHO) for *E. histolytica/dispar* (Singh et al. 2021).

Novel serological and molecular methods have recently emerged, demonstrating high sensitivity and specificity in distinguishing between *E. histolytica* and *E. dispar* (Fotedar et al. 2007). Newly developed techniques enable the differentiation of *E. histolytica* from *E. dispar* with heightened precision and accuracy. These methods involve the utilization of enzyme-linked immunosorbent assay (ELISA) to detect *E. histolytica* antigen, monoclonal antibodies that specifically recognize *E. histolytica* antigens for identification, and polymerase chain reaction (PCR) amplification of amoebic DNA for detection purposes (Gonin and Louise 2003, Redondo et al. 2006, Zeehaida et al. 2008, Wonsit et al. 1992, Fotedar et al. 2007). The latest strategies involve the simultaneous detection of *E. histolytica*, *E. dispar*, and *E. moshkovskii* through the implementation of multiplex nested PCR, enabling their discrimination.

The three species, *E. histolytica*, *E. dispar*, and *E. moshkovskii*, are frequently grouped together as the "*Entamoeba* complex" due to their similar cyst morphology, making it challenging to differentiate them. This resemblance often leads to an overestimation of the prevalence of amoebiasis, as it overlaps epidemiologically with the other two species (Yimer et al. 2017). In the previous chapter of our study, we collected a significant number of samples that showed the presence of microscopic *Entamoeba* organisms. Surprisingly, large a substantial number of samples tested negative for *E. histolytica* using PCR analysis. The abundance of these unidentified species suggests the possibility of other potential pathogens causing diarrheal infections.

Within the group of morphologically similar *Entamoeba* species, three species have been well characterized: *E. histolytica*, *E. dispar*, and *E. moshkovskii*. As already stated, these species share similar microscopic features, making it challenging to differentiate them based solely on

their appearance. Hence, it is essential to investigate the potential impact of these morphologically indistinguishable species on the occurrence of diarrheal case.

In this chapter, our primary objective is to identify *E. dispar* and *E. moshkovskii* among the unidentified species found in cases of diarrheal illness. To accomplish this, we will employ a PCR-based molecular method, which is a highly sensitive and specific technique for species identification. This method will allow us to accurately identify the presence of *E. dispar* and *E. moshkovskii* in the samples collected from individuals experiencing diarrheal symptoms.

Furthermore, we will assess the distribution patterns of these identified species in relation to the incidence of diarrheal cases. By examining their prevalence and potential association with diarrheal illness, we hope to gain insights into the pathogenic role of these species and their impact on public health.

4.2. Methodology

In the previous chapter (Chapter 3), we performed a species-specific PCR assay for *E. histolytica* on all 6051 samples. Out of these samples, only 52 tested positive for *E. histolytica*, while the specific species of *Entamoeba* could not be determined in a total of 203 samples (81.85%) that were positive under microscopy. To accurately determine the distribution of different species, we designed a set of primers that specifically target the *Entamoeba* genus. By conducting a genus-specific PCR, we were able to identify morphologically identical species within the genus. Subsequently, we performed a species-specific PCR to precisely identify the particular species present in the samples.

4.2.1. Study area and population

In the previous chapter, we mentioned that a total of 6051 diarrheal samples were collected from two hospitals in Kolkata: Infectious Disease & Beliaghata General Hospital and Dr. B C Roy Post Graduate Institute of Paediatric Sciences Hospital. These samples were obtained for the purpose of our present study and analysis.

4.2.2. Ethical statement

In accordance with the guidelines, the Institutional Human Ethics Committee of the ICMR-National Institute of Cholera and Enteric Diseases conducted a comprehensive review and granted ethical clearance for this study. The specific information regarding this approval can be found in the ethical statement section of Chapter 3, where detailed information is provided.

4.2.3. Sample collection and DNA extraction

In the preceding chapter, the collection procedure for samples from two hospitals was explained. Following their collection, the samples were promptly dispatched to laboratories for testing. The stool samples underwent microscopic examination in triplicate using saline and iodine wet mounts. Additionally, Trichome stain was employed. The presence of uninuclear, binuclear, trinuclear, or tetranuclear cysts or trophozoites of *Entamoeba* spp was observed and recorded. The findings and outcomes were detailed in the previous chapter.

Following the microscopic examination, DNA from each sample was extracted directly from the clinical samples using the STOOL DNA Minikit (QIAGEN, USA) according to the manufacturer's instructions. The isolated DNA was then subjected to genetic identification through PCR amplification using species-specific primers. To facilitate the molecular detection of parasites, Primer3 Software was utilized to generate the species-specific primers.

4.2.4. Primer design

A genus-specific primer set was developed for amplifying the 18S rRNA locus of the *Entamoeba* genus, referred to as the Genus-specific PCR assay. The forward and reverse primers for the *Entamoeba* genus were designed based on highly conserved regions within the 18S rRNA locus of five phylogenetically related Entamoeba species: *E. histolytica, E. dispar, E. moshkovskii, E. bangladeshi,* and *E. nuttalli*. The following sequences were analysed: *E. histolytica*, GenBank accession no. X56991 (1947 bp); *Entamoeba dispar*, GenBank accession no. Z49256 (1949 bp); *E. moshkovskii*, GenBank accession no. AF149906 (1944 bp); *Entamoeba bangladeshi*, GenBank accession no. KR025411 (1927 bp); *Entamoeba nuttalli*, GenBank accession no. AB485592.1 (2431 bp); *Entamoeba coli*, GenBank accession no. AF149914 (2101 bp); *Entamoeba chattoni*, GenBank accession no. AF149912 (1963 bp); *Entamoeba polecki*, GenBank accession no. AF149913 (1858 bp); *E. invadens*, GenBank accession no. AF149905 (1965 bp). The sequences were aligned using the ClustalW program (https://www.genome.jp/tools-bin/clustalw). Primer 3 online software was used to design the primers. Primer length and melting temperature were considered. Primer sequences specific

for *Entamoeba* spp. were as follows: EntaS_F: CTGCCAGTATTATATGCTGATGTT and EntaS_R: TCTCCTTCCTCTAAATAAGGAGATTTA.

4.2.5. Genus specific PCR assay

The genus-specific PCR assay was adjusted to ensure the amplification of each target. We performed PCR amplification in a final volume of 50 mL containing 1X PCR buffer and 1U of Biotaq DNA polymerase (Bioline, UK) to obtain the primary PCR product. After $MgCl_2$ concentrations ranging from 1.0 – 4.0 mM were checked, the optimal concentration of $MgCl_2$ was found to be 2.5 mM. 0.2 μM of each forward and reverse primer was found to be the optimal concentration for best results. After running a gradient PCR, the optimal annealing temperature was fixed to 55°C. The amplification was done in a thermal cycler as follows: 5 min at 94°C, followed by 35 cycles, each of 94°C for 45 sec, 55°C for 50 seconds and then 72°C for 50 seconds with a final extension at 72° C for 7 mins. Amplified PCR products were separated by agarose gel electrophoresis and visualised in a UV transilluminator after 0.5 μm/ml of ethidium bromide staining. The genus-targeted PCR products of *Entamoeba* spp showed 1803 bp - 2046 bp amplicon on the agarose gel, depending on the species.

To confirm the specificity of the genus-specific primer set in amplifying the 18S rRNA of the five *Entamoeba* species viz. *E. histolytica, E. moshkovskii, E. dispar, E. bangladeshi and E. nuttali*, genomic DNA preparations were utilized. The specificity of *E. moshkovskii* species-specific primer set was also assessed against DNA extracted from faecal samples of other pathogens, namely *E. histolytica, E. dispar, E. bangladeshi, E. nuttali, E. coli, G. lamblia*, *C. parvum*, *C. hominis*, *C. viatorum* and mixed bacterial infections. All the tested DNA samples were subjected to the abovementioned amplification protocol. The sensitivity of the nested PCR system was also evaluated using reference DNA templates by serial dilutions from 10 to 0.000019 ng/μL of DNA.

4.2.6. Species specific PCR assay

The positive samples obtained from the genus-specific PCR assay were subsequently employed for further identification of the specific *Entamoeba* species.

4.2.6.1 Identification of *E. histolytica*

In Chapter 3, the identification and prevalence of *E. histolytica* were already conducted using the EH1 forward primer and EH2 reverse primers. For more detailed information, please consult Chapter 3.

4.2.6.2. Identification of *E. dispar*

To ensure accurate amplification of the target region, the *E. dispar* species was identified using a nested primer set consisting of EDF (5'-AGTACAAAGTGGCCAATTTATGTAAG-3') and EDR (5'-ATTTTACTCAACTCTAGAGTTATGTG-3'). In order to assess the sensitivity of the primer set, reference DNA templates of the three *Entamoeba* species were utilized, with DNA concentrations ranging from 10 to 0.0013 ng/µL. The adjustments made in this *E. dispar*-specific PCR were aimed at optimizing the amplification process.

To obtain the nested PCR product, the positive products from the Genus-specific PCR were used as templates. For each reaction, 1 µl of the PCR products was utilized. The nested PCR amplification was conducted in a final volume of 50 µl reaction mixture, comprising 1X PCR buffer, 4.0 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of both forward and reverse primers (EDF and EDR), and 1U of Biotaq DNA polymerase (Bioline, UK). The reactions were carried out in a thermal cycler PCR system (Applied Biosystem).

The PCR reaction commenced with an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of 94°C for 40 seconds, 54°C for 25 seconds, and 72°C for 30 seconds. A final extension step at 72°C for 7 minutes concluded the PCR reaction. For the nested PCR, the concentration of MgCl2 used was 2.0 mM.

In each reaction, a previously known positive stool DNA sample for *E. dispar* was utilized as a positive control. The nested PCR procedure produced a 374 bp fragment when *E. dispar* was present. The amplified PCR products were separated by electrophoresis in a 1.5% agarose gel (Lonza SeaKem™ LE Agarose) using 1X Tris boric acid EDTA buffer. Subsequently, the gel was visualized under a UV transilluminator after staining with 0.5 µg/ml of ethidium bromide.

4.2.6.3. Identification of *E. moshkovskii*

The nested PCR system for *E. moshkovskii* employed the following primer sequences: EM_779bpNF (AACTAACGAAGGAGATGAAGTGAG) and EM_779bpNR

(GCCAGAGACATCGATTAAAATG). The sensitivity of the nested PCR system was assessed using reference DNA templates, with serial dilutions ranging from 10 to 0.000019 ng/µL of DNA. For the detection of *E. moshkovskii*, the primary PCR products underwent a second round of PCR amplification using a species-specific primer pair. In the nested PCR reaction, 1.0 µL of the primary PCR product was used as the template.

To obtain the nested PCR product, the amplification was carried out in a final volume of 50 µL reaction mixture, which included 1X PCR buffer, 4.0 mM MgCl2, 0.2 mM of each dNTP, 0.2 µM of both the forward and reverse primers (EM_779bpNF & EM_779bpNR), and 1U of Biotaq DNA polymerase (Bioline, UK). The reactions were performed using a thermal cycler PCR system (Applied Biosystem). The PCR reaction for the nested PCR began with an initial denaturation step at 94°C for 3 minutes. It was then subjected to 35 cycles of 94°C for 40 seconds, 57°C for 35 seconds, and 72°C for 45 seconds. This was followed by a final extension step at 72°C for 7 minutes. The nested PCR was conducted using 2.0 mM MgCl2. In the presence of *E. moshkovskii*, the nested PCR generated a 779 bp fragment. To serve as a positive control, genomic DNA of *E. moshkovskii* isolated from an Axenic culture (specifically, an isolate from Thailand) was obtained as a gift from Dr. Seiki Kobayashi at the School of Medicine, Keio University.

The amplified PCR products were separated by electrophoresis in a 1.5% agarose gel (Lonza SeaKem™ LE Agarose) using 1X Tris boric acid EDTA buffer. After staining with 0.5 µg/ml of ethidium bromide, the gel was visualized under a UV transilluminator. The presence of *E. moshkovskii* was indicated by the appearance of a 779 bp fragment on the gel.

4.2.6.7. Statistical Analysis

Statistical data analysis was performed using GraphPad Prism version 8.4.2, developed by GraphPad Software in California, USA.

4.3. Results

4.3.1. *Entamoeba* species burden in diarrhoeal patients

In a previous chapter, initial examination of 6051 samples using a light microscope (**Fig. 4.1** for microscopic view) revealed that 4.10% (n/N=248/6051, 95% CI 0.036-0.046) contained cysts or trophozoites of *Entamoeba* spp. To enhance the accuracy of detection, a genus-specific PCR assay was performed on all the samples. This molecular technique (**Fig. 4.2**) identified

4.84% (n/N=293/6051, 95% CI 0.043 - 0.054) of the samples as positive for *Entamoeba* spp. Interestingly, the PCR assay demonstrated that 15.36% (n/N=45/293, 95% CI 0.273-0.380) of the samples positive for *Entamoeba* spp. were not initially detected using microscopy.

In order to explore the presence of specific *Entamoeba* species, specifically *E. moshkovskii* and *E. dispar*, a subsequent round of nested PCR was conducted on the samples that exhibited positive results in the initial genus-specific PCR assay. This additional molecular test was specifically designed to identify and screen for the presence of *E. moshkovskii* and *E. dispar*.

4.3.2. *Prevalence of E. histolytica*

The overall prevalence of *E. histolytica* was found to be 0.86%. A details of the prevalence results can be found in the results section of Chapter 3, specifically under the subheading "Prevalence of *E. histolytica*."

4.3.3. *Prevalence of E. dispar*

Out of the 6051 diarrheal samples that were screened, a total of 8 positive cases of *E. dispar* infection were detected, constituting a prevalence rate of 0.13% (n/N = 8/6051, 95% CI 0.0006 to 0.0027) among the overall infections. Among the 8 cases of *E. dispar* infection, 5 cases (62.5%) were observed in male patients, while the remaining 3 cases (37.5%) were observed in female patients. Among the cases of *E. dispar* infection, 37.5% (3 cases) were observed in the age group of 0-5 years. An equal distribution of 25% (2 cases for each age group) was found in both the age groups of 5-12 years and 30-50 years. The remaining 12.5% (1 case) of cases belonged to the age group above 50 years. It is important to note that due to the limited number of positive *E. dispar* cases, statistical analysis could not be performed.

4.3.4. *Prevalence of E. moshkovskii*

The PCR system used in the prevalence study of *E. moshkovskii* (refer **Fig. 4.1** for microscopic view and **Fig. 4.2** for molecular identification) was capable of identifying as little as 1.2 pg of genomic DNA from *E. moshkovskii*. The prevalence of *E. moshkovskii*, determined through nested PCR assay, was found to be 3.12% (n=189/6051, 95% CI 0.027-0.036). These results have increased attention towards studying *E. moshkovskii* infection. Further investigation of the microscopic data revealed that 19.04% (n/N=36/189, 95% CI 0.141-0.252) of *E. moshkovskii* infections were missed using microscopy but were later identified using the nested PCR test. This finding suggests that *E. moshkovskii* infections produce fewer cysts compared

to other *Entamoeba* species. Out of the 189 samples that tested positive for *E. moshkovskii* via PCR, only 9.00% (n/N=17/189, 95% CI 0.056-0.14) showed the presence of trophozoites through microscopy.

There was a higher occurrence of *E. moshkovskii* in males (*E. moshkovskii:* n/N = 117/3354, 61.35%, 95% CI 0.98-1.80) compared to females (*E. moshkovskii:* n/N = 72/2697, 2.67%); however, the difference between the two groups was not found to be statistically significant (P=0.073). On the other hand, the presence of infections (Sole) was significantly associated with different age groups. The highest likelihood of acquiring an infection was observed among individuals in the 5-12 age group (p value=0.0003), while the lowest likelihood was seen in the 19-29 age group (**Table 4.1)**.

Table 4.1 Multinomial Logistic Regression Models examining gender and age groups as significant risk factors for *E. moshkovskii* infection (statistical significance assessed via Fisher's exact test with p < 0.05).

Category	**Group**	**% of positive case**	**OR**	**95% CI**	**P value**
Gender	Male	3.49%	1.32	0.98-1.80	0.073
	Female	2.67%	-	-	-
Age	0-4 yrs	1.19%	0.26	0.15-0.43	<0.0001*
	5-12 yrs	9.12%	2.19	1.44-3.33	0.0003**
	13-18 yrs	5.61%	1.74	1.12-2.74	0.019
	19-29 yrs	0.92%	0.20	0.09-0.46	<0.0001*
	30-50 yrs	2.11%	0.47	0.28-0.79	0.0057*
	>50	4.39%	1	-	-

**positively associated with the age group, *negatively associated

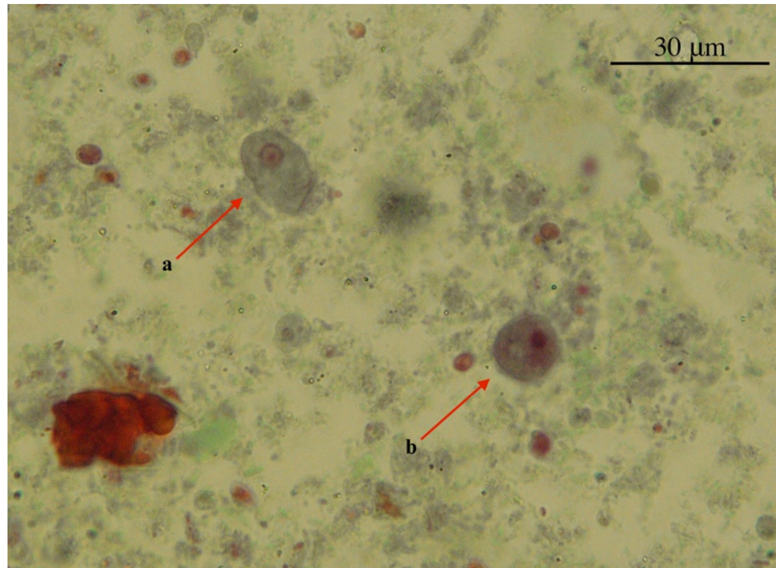


Fig. 4.1 Microscopic view of *Entamoeba moshkovskii* cysts and trphozoites after Trichrome staining (40X). a. Trophozoites of *Entamoeba moshovskii.;* b. Cyst of *Entamoeba moshovskii*

4.3.5. Prevalence of unidentified *Entamoeba* species

Out of the total samples tested (N=6051), 44 samples showed a positive result for the *Entamoeba* genus (n/N= 44/6051, 0.73%, 95% CI 0.0054 to 0.0098), but did not belong to the known species *E. histolytica, E. dispar,* or *E. moshkovskii*. It is possible that this significant number of positive samples may be attributed to phylogenetically related species such as *E. bangladeshi* or other unidentified species within the *Entamoeba* genus. Although the genus-specific primer used in this study can also amplify *E. nuttalli*, it is important to note that *E. nuttalli* is not a well-documented human commensal. Therefore, it is necessary to conduct further investigations in order to characterize these numerous unrecognized *Entamoeba* species found in the study area.

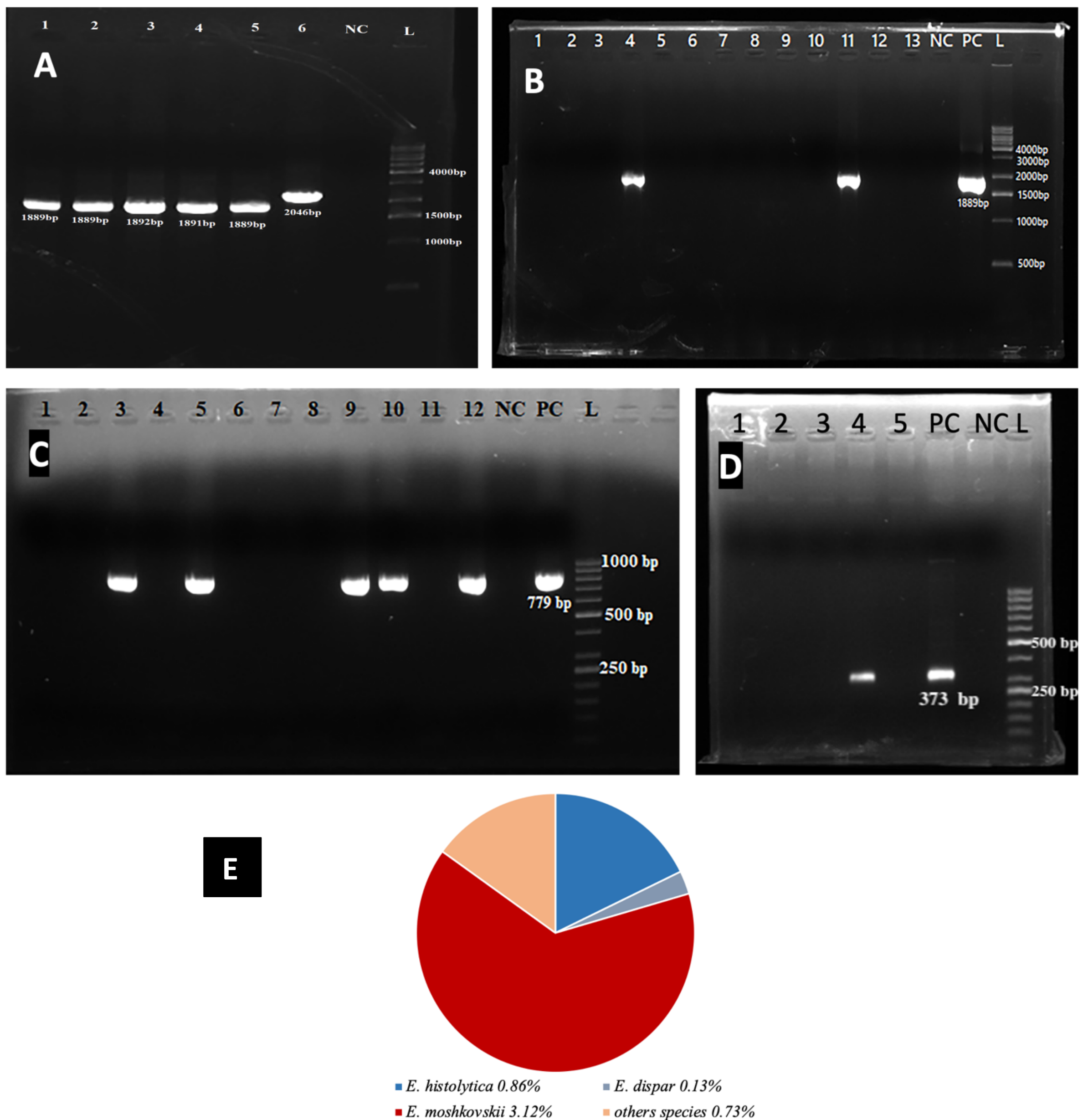


Fig. 4.2. A. Amplification of 18SrRNA locus of different *Entamoeba* species using genus-specific primer set; *1: E. histolytica 1889bp, 2. E. moshkovskii 1889bp, 3: E. dispar 1892bp, 4: E. bangladeshi 1891bp, 5. E. nuttali 1889bp, 6. E. coli* 2046bp. NC: Negative control *; L:* Ladder. **B.** Amplification of 18SrRNA locus of *Entamoeba* spp using genus-specific primer set. PCR product size:1889bp-2046bp depends on species present in stool sample. Lanes 1-13 are diarrhoeal stool samples collected in this hospital based surveillance study. PC: Positive control contains *E. moshkovskii* DNA, NC: Negative control, Lane- 4 and 11 are positive for *Entamoeba* spp. **C.** Amplification of 18SrRNA locus of *Entamoeba moshkovskii* using nested PCR assay. Nested PCR product size: 779bp. Lanes 1-13 contains primary PCR product of diarrhoeal stool samples collected in this hospital based surveillance study. 14: Positive control contains primary PCR product of *E. moshkovskii* DNA, 15: Negative control, Lane- 2 and 6 are positive for *Entamoeba moshkovskii* spp. **E.** Amplification of *E. dispar* positive sample. **D.** Prevalence of different *Entamoeba* species.

4.4. Discussion

The study was conducted to determine the prevalence of common *Entamoeba* species in stool samples from diarrheal patients admitted to the Infectious Disease Hospital and B C Roy Hospital in Kolkata. This is the first study conducted in Eastern India that utilizes molecular biological techniques to report the prevalence of *Entamoeba moshkovskii* in clinical stool samples. According to an active surveillance study on the detection of common enteric parasites conducted over the past two decades, a decreasing trend in *E. histolytica* infection has been observed in the Kolkata area in recent years (Mukherjee et al. 2009; Karmakar et al. 2017; Mukherjee et al. 2017). Additionally, our earlier chapter has reported non-seasonal sporadic infections with *E. histolytica*, which is unusual for a tropical area like Kolkata (Mukherjee et al. 2009; Mukherjee et al. 2017). Microscopic examination of diarrheal stool samples has revealed the presence of amoeba cysts/trophozoites that exhibit morphological features similar to *E. histolytica* in a significant proportion throughout the year in Kolkata. After performing PCR based molecular identification, it was found that the most abundant species of *Entamoeba* observed in diarrheal stool samples in Kolkata is a morphologically indistinguishable amoeba from *Entamoeba histolytica* and is a related species called *Entamoeba moshkovskii*. A recent study in Egypt showed that 85% of amoebic infections were caused by so called non-pathogenic *Entamoeba* spp. such as *E. dispar*, *E. moshkovskii*, *and E. hartmani* (Abozahra et al. 2020). This observation has led to an increased interest in the study of traditionally non-pathogenic *Entamoeba* species. Human are a true host for amphizoic amoeba *E. moshkovskii* (Shimokawa et al. 2006). Moreover, recent evidence from different studies supports the pathogenicity of *E. moshkovskii* (Ali et al. 2003; Shimokawa et al. 2006; Parija and Khairnar 2005; Kyany'a C et al. 2019). Although *E. moshkovskii* is identified as a cause of human infection, endemicity has not been appropriately assessed in most epidemiological studies (Samie et al. 2020). In this study, we employed microscopic and molecular tools to determine the prevalence and genetic structure of *E. moshkovskii* in and around Kolkata.

The present study reported that *Entamoeba* spp was prevalent in diarrhoeal stool samples and other enteric pathogens in Kolkata. More than half of the amoebic infection was caused by *Entamoeba moshkovskii*. This finding is alarming as it implies that *E histolytica* infections previously decreased and *E. moshkovskii* has been taking its place. Although *E. moshkovskii* was highly prevalent in diarrheal patients, we did not find any hematophagous trophozoites of *E. moshkovskii* during microscopy, indicating its non-invasive nature. These results will boost research for a better understanding of the mechanism of pathogenicity in the parasite. The raw

data revealed that a more significant proportion of men were infected with *E. moshkovskii* than women. Still, the difference was not statistically significant, which indicates that the probability of infection with *E. moshkovskii* is equal for both genders. Our data revealed that *E. moshkovskii* infections were most predominant in age 5-12. The higher prevalence of *E. moshkovskii* in this age group might be associated with their lack of health education regarding hygiene practices. A recent study in Bangladesh reported that 21% of children aged 2–5 were infected with *E. moshkovskii*, which was associated with diarrhoea (Ali et al. 2003). Many other studies also reported *E. moshkovskii* as an enteropathogen in patients suffering from diarrhoea or dysentery (Ali et al. 2003; Shimokawa et al. 2006; Parija and Khairnar 2005; Kyany'a C et al. 2019). In a study conducted by Parija and Khairnar in 2005, it was demonstrated that relying solely on microscopic findings for a diagnosis led to the misdiagnosis of 81% of suspected infections (Parija and Khairnar 2005). Similarly, the study of our previous chapter revealed that 81.85% of samples, which tested positive for *Entamoeba* spp. through microscopy, did not actually contain *E. histolytica*. In the present chapter, we have observed that a significant portion of these unidentified species corresponds to *E. moshkovskii*. The study focused on patients presenting with diarrheal symptoms, and a significant number of positive cases of *E. moshkovskii* suggest that this particular organism may have a more active role than previously believed in the human gut. Rather than being solely a harmless commensal, it appears that *E. moshkovskii* could potentially act as a pathogen, leading to the development of diarrhoea and other gastrointestinal disorders in the studied population.

This study has revealed a considerable number of positive cases of *E. dispar*. The presence of *E. dispar*, emphasizing the need for accurate diagnosis of diarrheal patients to screen for *Entamoeba* species. Failing to do so may result in misdiagnosis or inappropriate treatment, including the unnecessary use of anti-amoebic drugs. In addition, the study highlights the presence of 0.73% unidentified *Entamoeba* species, emphasizing the importance of employing molecular techniques such as PCR and DNA sequencing for their accurate characterization. The results of this study also indicate that a considerable proportion of these unidentified species may be attributed to *E. bangladeshi*. This is because our genus-specific primer only identifies phylogenetically close species of *E. histolytica*. Therefore, further research should be conducted to assess the role of these unidentified species, particularly *E. bangladeshi*, in the incidence of diarrheal diseases.

In this particular region, one of the most prevalent species responsible for amoebic infections is *E. moshkovskii*. However, there is a significant lack of research focusing on the genetic and phylogenetic aspects of *E. moshkovskii* in India, leaving much of its characteristics unknown. In the upcoming chapter, we aim to fill this gap by conducting a thorough characterization of *E. moshkovskii* isolates in eastern India. Additionally, we will assess its potential role as a pathogen in causing diarrheal incidents in human.

4.5. Conclusion

This study has revealed a significant increase in the infection of *E. moshkovskii* among individuals suffering from diarrhoea in and around Kolkata. This higher prevalence rate suggests a potential involvement of *E. moshkovskii* in causing diarrheal diseases, alongside other pathogenic species like *E. histolytica*. Consequently, it is essential to thoroughly investigate the potential contribution of *E. moshkovskii* to the incidence of diarrhoea. This discovery is particularly concerning as it suggests a shift in the pattern of infections, with *E. moshkovskii* potentially replacing *E. histolytica* as a prominent causative agent.

CHAPTER V

GENETIC CHARACTERIZATION OF *E. MOSHKOVSKII* ISOLATES BASED ON SMALL RIBOSOMAL RNA LOCUS

Genetic characterization of *E. moshkovskii* isolates based on small ribosomal RNA locus

5.1. Background

The pathogenic potential of *E. moshkovskii* in humans remains a captivating yet enigmatic subject within the field of amoebic infections. Our examination in chapter 4 unveiled that *E. moshkovskii* is conspicuously prevalent in Kolkata and its neighboring areas, establishing its prominence as a species frequently associated with amoebic infections in this region. However, a notable scarcity of research exists concerning the genetic and phylogenetic dimensions of *E. moshkovskii* in India, leaving numerous aspects of its characteristics shrouded in mystery.

Amoebiasis, an infection by enteric protozoa, most commonly *Entamoeba histolytica* is globally considered a potentially severe and life-threatening infection (WHO 1997). Together with the pathogenic species *Entamoeba histolytica,* the genus *Entamoeba* includes many other species that reside in the human intestinal lumen, namely *E. dispar, E. moshkovskii, E. bangladeshi, E. polecki, E. coli,* and *E. hartmanni* (Fotedar et al 2007). In many cases, infections with the trophozoites of *Entamoeba* spp. can result in harmless colonization in the intestinal lumen of asymptomatic carriers, passing cysts in their stool (non-invasive infection). In other cases, the trophozoites invade the intestinal mucosa (intestinal disease) and, through the bloodstream, reach extraintestinal sites in other tissues such as the liver, brain, and lungs (extraintestinal disease) with consequential pathologic manifestations (Espinosa-Cantellanoa and Martínez-Palomo 2000). Abdominal pain, diarrhoea, nausea, vomiting and flatulence are the acute symptoms of amoebiasis caused by the eukaryotic parasite *Entamoeba histolytica* (Espinosa-Cantellanoa and Martínez-Palomo 2000, Pritt and Clark 2008, Ali 2015]. Other *Entamoeba* species are mostly commensals or are said to rarely infect humans (Heredia et al. 2007).

Amoebiasis is typically diagnosed through the use of light microscopy to examine wet smears or stained stool samples (Hamzah et al. 2007). While this method is low-cost and straightforward, its effectiveness is limited as it cannot distinguish between the cysts and trophozoites of the pathogenic species *Entamoeba histolytica* and the non-pathogenic species like *Entamoeba dispar, Entamoeba bangladeshi* (a newly identified species), and the amphizoic amoeba *Entamoeba moshkovskii*, which sporadically infects humans (Fotedar et al. 2007, Hamzah et al. 2006). These four species are genetically related and morphologically

indistinguishable with different biochemical features (Fotedar et al. 2007) Amoebiasis affects approximately 50 million people in tropical regions and nearly 100,000 deaths are reported annually (Tengk et al. 2011). After malaria and schistosomiasis, it is the third foremost parasitic cause of death in humans Singh et al. 2014). Although all deaths could be due to invasive *E. histolytica* infestation, the prevalence is overestimated due to its epidemiological overlap with other morphologically indistinguishable species, specifically *Entamoeba dispar* and *Entamoeba moshkovskii* (Yimer et al. 2017). Moreover, cysts of another nonpathogenic amoeba, *Entamoeba hartmanni* can be mystified with *E. histolytica* under *the* microscope which is even smaller in size (> 10 μm) (Gomes et al. 2014, Burrows et al. 1059). In many geographical areas, the actual prevalence of each species is not well characterized particularly for *E. moshkovskii,* as some reports suggest that it has a potential role in provoking human disease (Khairnar and Parija 2007). So, it is important to understand the molecular epidemiology of *E. moshkovskii* in endemic countries in the study of amoebiasis and it has become crucial in the last decade.

Entamoeba moshkovskii was first described as a distinct species from Moscow by Tshalaia in 1941. It was primarily considered to be a free-living environmental strain and still regarded as a common protozoan species found in anoxic sediments to brackish coastal pools. It is osmotolerant in nature and can be cultured in various media suitable for intestinal protozoa, in which it grows easily at temperatures of 10-15° C and 37° C (Clark and Diamond 1991). Although all the characteristics differentiate *E. moshkovskii* from *E. histolytica* and *E. dispar*, the entire life-cycle, including excystment and metacystic development, closely resemble *E. histolytica* and *E. dispar*. Size of the amoeba trophozoites varies from 10 to 120µ, with an average of 25µ and cysts vary from 5 to 16 µ, with an average of 10µ (Clark and Diamond 1991). In 1961, an *E. histolytica*-like strain was obtained from a resident of Laredo, Texas, who suffered from diarrhea, weight loss, and epigastric pain and the strain was named *E. histolytica* Laredo strain which shared many biological characteristics with *E. moshkovskii*. Both the Laredo strain and *E. moshkovskii* grew easily at room temperature, were osmotolerant, and resistant to drugs used in the chemotherapy of amoebiasis, for instance, emetine (Dreyer 1961). Subsequent molecular studies revealed that the *E. histolytica* Laredo strain is *E. moshkovskii,* the first human isolate of *E. moshkovskii* (Dreyer 1961, Diamond and Bartgis 1970).

Nowadays importance of *E. moshkovskii* is increasing in the study of amoebiasis, and it is reported as a common *Entamoeba* infection in humans in some settings. Colonization of *E. moshkovskii* in human hosts has been reported in countries such as the United States, Italy,

Iran, Turkey, Bangladesh, India (Pondicherry), Kenya, Australia, Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil (Ngui et al. 2012; Ali et al. 2003). Most of the stool samples in these studies were submitted to clinical microbiology laboratories from patients with gastrointestinal complications, indicating that *E. moshkovskii* might be associated with pathogenicity. In India (Pondicherry), it is reported that *E. moshkovskii* cannot invade intestinal mucosa and does not have any ingested erythrocytes, unlike that *E. histolytica* (Parija and Khairnar 2005). In HIV-1-infected persons in northern Tanzania, *E. moshkovskii* is also not associated with clinical indicators. But in Bangladesh, this species has been identified as the only likely pathogen in individuals with gastrointestinal clinical manifestations, including dysentery (Shimokawa et al. 2012). However, in these patients, no studies of viral or bacterial agents were conducted to rule out other pathogens or potential pathogens. Another study in Bangladesh by Shimokawa et al. 2012 also pointed out a possible cause of diarrhoea in infants, which was due to *E. moshkovskii* infection (Shimokawa et al. 2012). While in Malaysia, it was isolated from both symptomatic and asymptomatic cases (Anuar et al. 2012). In the murine model of intestinal amebiasis, *E. moshkovskii* also caused diarrhoea, weight loss, and colitis (Shimokawa et al. 2012). Thus, the pathogenicity of *E. moshkovskii* in humans remains unclear.

For parasite identification, phylogeny and genetic characterization, small subunits of nuclear ribosomal RNA (18S rRNA) loci are recognized as potential targets (Stensvold et al. 2011; Haghighi et al. 2003; Haghighi et al. 2008; Dong et al. 2017). Therefore, amplification and sequencing of this gene is being extensively used for decades (Al-Areeqi et al. 2017; Fotedar et al. 2007). Moreover, genetic variations based on 18S rRNA can be significant in pathogenicity for parasites.

In the previous part of our study, we successfully identified a significant number of *E. moshkovskii* positive samples in diarrheal cases. However, we were unable to confirm the pathogenicity of *E. moshkovskii* solely based on its presence in stool samples. Therefore, the primary objective of this chapter was to characterize the *E. moshkovskii* isolates using 18S rRNA analysis. Additionally, we conducted screenings for other diarrheagenic organisms in the *E. moshkovskii* positive samples to gather epidemiological evidence regarding the pathogenic potential of *E. moshkovskii*. Furthermore, we explored into the population structure of this organism to gain a better understanding of its characteristics.

5.2. Methodology

5.2.1. DNA Sequencing

The 18S rRNA locus of the positive samples was sequenced to characterize the local isolates of *E. moshkovskii*. As the same-size amplified products do not unavoidably mean identical DNA sequences, we directly sequenced the PCR products without cloning them into any vector to reduce the chances of any sequence selection. For DNA sequencing of *E. moshkovskii* positive samples obtained in this study were amplified separately using ExTaq DNA polymerase (Takara, Japan). We used ExTaq because of its higher fidelity than standard Taq with a lower mutation rate. The aforementioned species-specific primer set was employed directly to amplify the 18S rRNA locus of the positive samples by conventional PCR method. PCR was accomplished in a 50 µL reaction mixture containing: 0.50 µL Takara Ex Taq, 5 µL 10X Ex Taq Buffer, dNTP mixture 3 µL, 0.2 µM of each forward and reverse primer (EM_779bpNF & EM_779bpNR), approximately 200 ng stool DNA and nuclease-free water (Ambion™) up to 50 µL. The reaction mixture was subjected to an initial denaturation step at 94°C for 5 mins, followed by 35 cycles of 60 s at 94°C of denaturation, primer annealing for 40 s at 57°C and extension for 55 s at 72°C. A final seven minutes polymerization step at 72°C was also performed. Successfully amplified PCR products were purified using the Roche PCR Gel extraction Kit as per the manufacturer's protocol. The purified PCR products were sequenced using the standard BigDye terminator V3.1 sequencing kit (Applied Biosystem, USA) following the manufacturer's instructions. Sequencing was performed with a 5730 DNA analyzer (Applied Biosystem, Foster City, CA, USA). The accuracy of the sequence was verified with sequencing in the 5' - 3' direction using both forward and reverse primer separately.

5.2.2. Sequence alignment, nucleotide polymorphisms analysis

The obtained 18S rDNA gene sequences of *E. moshkovskii* compared to those available in the GenBank database using the BLAST tool (NCBI - https://blast.ncbi.nlm.nih.gov/Blast.cgi). All of the obtained sequences were deposited in NCBI GenBank (accession numbers ON965383-ON965450). Multiple alignments of the nucleotide sequence allowed us to analyze nucleotide sequence variations. The obtained sequences were aligned using ClustalW multiple sequence alignment program of GenomeNet Bioinformatics tools and edited manually. This alignment was also performed with MultAlin using identity parameter values of -1 and -0 and the penalty

default values to determine sequence variations. All obtained sequences were aligned and adjusted in MEGA X. Substitution matrix (Maximum likelihood/ML) and transition/transversion (ML) bias were estimated using the same software. In the substitution matrix (ML), each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution patterns and rates were estimated under the General Time Reversible model (+G+I) (Kumar et al. 2008). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 200.0000). The rate variation model allowed some sites to be evolutionarily invariable ([+*I*], 0% sites). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, the sum of *r* values is made equal to 100. For estimating Maximum likelihood values, a tree topology was automatically computed (Kumar et al. 2008). This analysis involved 68 nucleotide sequences. For transition/transversion (ML) bias determination substitution patterns and rates were estimated under the Tamura-Nei (1993) model (+G+I). There were a total of 733 positions in the final dataset. Nucleotide composition, parsimony-informative sites, singleton sites, variable sites (S), the number of haplotypes (h), haplotype diversity (Hd), the average number of nucleotide differences (K), and nucleotide diversity (π) were determined from the aligned sequences using the program DnaSP v5.

5.2.3. Genetic structure analysis

Partitions of genetic diversity within and among different population subdivisions of local isolates obtained from the present study were calculated using Wright's F statistics (F_{ST}). Population subdivision was performed based on the co-infection status of the isolates. F_{ST} is a measure of genetic differentiation among populations. DnaSP v5.0 package was used to estimate the mean pairwise differences between the populations. The significant difference in F_{ST} was from 0 based on 1000 random permutations of the dataset. Four neutrality tests using Tajima's D, Fu's FS, Fu and Li's D and F statistics were also performed through DnaSP v5.0 for assessing the probable population expansion.

5.2.4. Phylogenetic Analysis

The maximum Likelihood method and Tamura-Nei model were applied to infer the evolutionary history (Tamura and Nei 1993). The tree with the highest log likelihood (-

6543.31) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [8 categories (+*G*, parameter = 200.0000)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.42% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 76 nucleotide sequences. There were a total of 822 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2008, Felsenstein et al. 1985).

5.2.5. Haplotype network construction

The relationship between the haplotypes of *E. moshkovskii* in different coinfected subgroups was inferred by constructing a Median Joining haplotype network in PopART v1.7. The isolates were colour-coded according to specific coinfection groups to derive a relationship with sequenced data.

5.2.6. Data collection of other Enteropathogen infections

Entamoeba histolytica, Cryptosporidium, and *Giardia* were identified by PCR method as described elsewhere (Mukherjee et al. 2009). Helminth parasites were detected by light microscopy after wet mount. Other enteric pathogens like *Vibrio cholera* O1/O139, *Salmonella* spp., *Shigella* spp. *Campylobacter jejuni,* Rotavirus, astrovirus and adenovirus co-infection information with *E. moshkovskii* were obtained from the institutional database of ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India.

5.2.7. Geographical distribution of *E. moshkovskii* infection

The Choropleth Mapping was used to construct a GIS map of *E. moshkovskii* prevalence for all patients enrolled in the surveillance system. The geographical locations of the patients were obtained from the hospital case record file (CRF). This mapping was employed to evaluate the spatial distribution of *E. moshkovskii* in the catchment area. The map was created with the free license of the Datawrapper tool. If patients from any area comprised less than 1% of the total sample size, the site has been excluded from the GIS mapping study.

5.2.8. Cultivation of *Entamoeba* spp.

In this study, we used Trophozoites of the *E. moshkovskii* (An isolate of Thailand), a gift from Dr Seiki Kobayashi, School of Medicine, Keio University. The *E. moshkovskii* strain was regularly cultivated in our lab using the BI-S-33 medium and maintained at a temperature of 25°C. Trophozoites under the log phase of growth were used in the experiments as a positive control.

5.2.8.1 *Preparation of BIS-33 medium*

The culture medium Biosate-Iron-Serum (BI-S-33) is designed for cultivating *E. moshkovskii* and consists of an axenic medium supplemented with 10% heat-inactivated adult bovine serum (Deloer et al. 2016). Refer to **Table 5.1** and **Table 5.2** for the detailed composition.

Table 5.1 Composition of Biosate-Iron-Serum (BI-S-33) medium.

	Reagent	**Amount (g/880 ml)**
Mixture A	Biosate	30.0
	D-glucose	10.0
	Sodium chloride (NaCl)	2.0
	Potassium phosphate (KH_2PO_4)	0.6
	Dipotassium hydrogen phosphate (K_2HPO_4)	1.0
	L-Cysteine	1.3
	L-Ascorbic acid	0.2
	Ferric ammonium citrate	0.0228
	ddH_2O	X ml[a]
[a]Subtotal (Mixture A)	880 ml	
	#Mixture A	880 ml
	#ABS (adult bovine serum)	150 ml
	#Vitamin mixture	20 ml
	#Penicillin–Streptomycin (each 10,000 U/ml)	14 ml
Total	1064 ml	

#After cooling the media (Mixture A), heat-inactivated adult bovine serum, a vitamin mixture, and antibiotics are added. Penicillin–streptomycin is generally used. Foetal bovine serum is not acceptable because fetuin is toxic to the parasite.

[a] Firstly 600 mL ddH_2O should be taken in a beaker. Then all ingredients of mixture A should be added to the beaker. Next, add water up to 880 mL and adjust the pH to 6.8. This mixture needs to be autoclaved at 121 °C for 15 min.

Table 5.2 Composition of vitamin mixture added in BI-S-33medium.

	Reagent	**Amount (mg)**
A 25 ml ddH_2O	Niacinamide	45
	Pyridoxal HCI	4
	Calcium pantothenate	23
	Thiamine hydrochloride	5
	Vitamin B12	1.2
B 45 ml ddH_2O (#1)	Riboflavin	7
C 45 ml ddH_2O (#1)	Folic acid	5.5
D 45 ml ddH_2O	d-Biotin	2
Solution 1 (A + B + C + D)		160 ml
E 5 ml 95% ethanol		1 mg
F 30 ml ddH_2O	Tween 80	35 ml
ddH_2O		X
Total (Solution 1 + 2 + ddH2O)		200 ml

#1 0.1 N NaOH is used to dissolve Riboflavin (B) and Folic acid (C) in ddH_2O (10 mL) and the final volume of them is 45 ml each.

5.2.9. Intracecal inoculation of *Entamoeba* spp. to assess pathogenic potential

Male CBA/J mice were selected as the animal model and were maintained in a controlled environment free from pathogens. The mice were subjected to the experimental procedure at the age of 5-8 weeks. The research took place at the NIID in Tokyo, Japan, as a collaborative study and received approval from the animal ethical review board at NIID, Tokyo, Japan.

Trophozoites of *E. histolytica* HM1:IMSS and *E. moshkovskii* (strain of Thailand) were obtained from culture tubes. The tubes were placed on ice for 10 minutes to facilitate trophozoite collection. After that, the trophozoites were collected, and their number was determined. The experiment commenced by anaesthetizing the mice and removing the fur from their abdominal area. A small incision was made in the skin, exposing the cecum. Then, 150 μL of 1 × 106 trophozoites from both *E. histolytica* HM1:IMSS and *E. moshkovskii* (a strain of Thailand) were injected into the proximal, middle, and apical sections of the cecum. Afterwards, the cecum was gently blotted, and the peritoneum and skin were sutured to close the incision. To maintain the body temperature of the mice, they were placed on warming blankets set at 37°C throughout the procedure. For the study, a total of 9 male mice were chosen and divided into three groups, each containing three mice. Two groups of mice were intentionally infected with the same number of trophozoites from two distinct strains of *Entamoeba histolytica* (HM1:IMSS) and *Entamoeba moshkovskii*, respectively. The third group of mice was not infected with any *Entamoeba* species and served as the negative control.

Following a 4-day intracecal challenge, the mice were euthanized, and their ceca were collected and preserved in glutaraldehyde diluted in PBS (pH 7.2). The glutaraldehyde solution was initially warmed at 37°C and then incubated for 1.5 hours at room temperature. For scanning electron microscopy, the dehydration process commenced with 50% and 70% ethanol, after which the membrane was detached from the support ring. Dehydration was then carried out using a series of alcohol baths with increasing concentrations. Subsequently, the sections underwent critical-point drying using CO2 or chemical treatment with HMDS (Hexamethyldisilazane). Once dried, the samples were affixed to specimen stubs using carbon-conductive tabs. Prior to examination, the tissue cells containing amoebas were coated with a layer of gold.

5.2.9. Statistical Analysis

GraphPad prism v.8.4.2, CA, USA, was used to analyze the data. The relationship between the prevalence of *E. moshkovskii* with others variables like age, gender, and other co-infections/additional enteropathogens was measured by testing X^2. Fisher's exact test was applied to evaluate the statistical association between gender and the occurrence of infection of *E. moshkovskii.* Two-way ANOVA was employed in three-year sample comparisons to assess the statistical significance of the seasonal pattern of *E. moshkovskii* infection. In all cases, a p-value less than 0.05 was considered significant.

5.3. Results

5.3.1. Additional enteropathogens in stool samples of *E. moshkovskii* (sole) infected peoples

Many bacteria, viruses, enteric parasites, and helminths are associated to induce diarrhoea. The coinfection status with other diarrheagenic organisms was analyzed by examining the data from our institutional database, followed by evaluating the statistical significance. In the 189 diarrheal stool samples with *E. moshkovskii,* few were coinfected with one kind of enteropathogen, like 5.82% (n/N=11/189, 95% CI 0.032-0.102) with *E. histolytica*, 2.12% (n/N=4/189, 95% CI 0.008-0.053) with *G. lamblia*, 3.17% (n/N=6/189, 95% CI 0.015-0.068) with *Cryptosporidium* spp, 7.94% (n/N=15/189, 95% CI 0.049-0.127) with Soil-transmitted helminths (STH), 12.70% (n/N=24/189, 95% CI 0.087-0.182) with *E. coli*, 10.58% (n/N=20/189, 95% CI 0.007-0.158) with *Shigella* spp, 4.23% (n/N=8/189, 95% CI 0.022-0.081) with *V. cholerae*, 2.12% (n/N=4/189, 95% CI 0.008 0.053) with Rotavirus (Fig 5.1). 4.76% (n/N=9/189, 95% 0.025-0.088) samples were co-infected with 2 or >2 enteropathogens. 46.56% (n/N=88/189, 95% CI 0.396-0.537) of the samples were positive solely for *E. moshkovskii* (Fig 5.1). So it is, therefore, worthy of note that the diarrheal cases associated with *E. moshkovskii* were not commonly coinfected in Kolkata. Many positive samples were infected with *E. moshkovskii,* and the sole infection with *E. moshkovskii* was significantly associated with diarrheal incidence (X^2= 335.5, df=9; $P<0.0001$) in this study area (**Fig 5.1**).

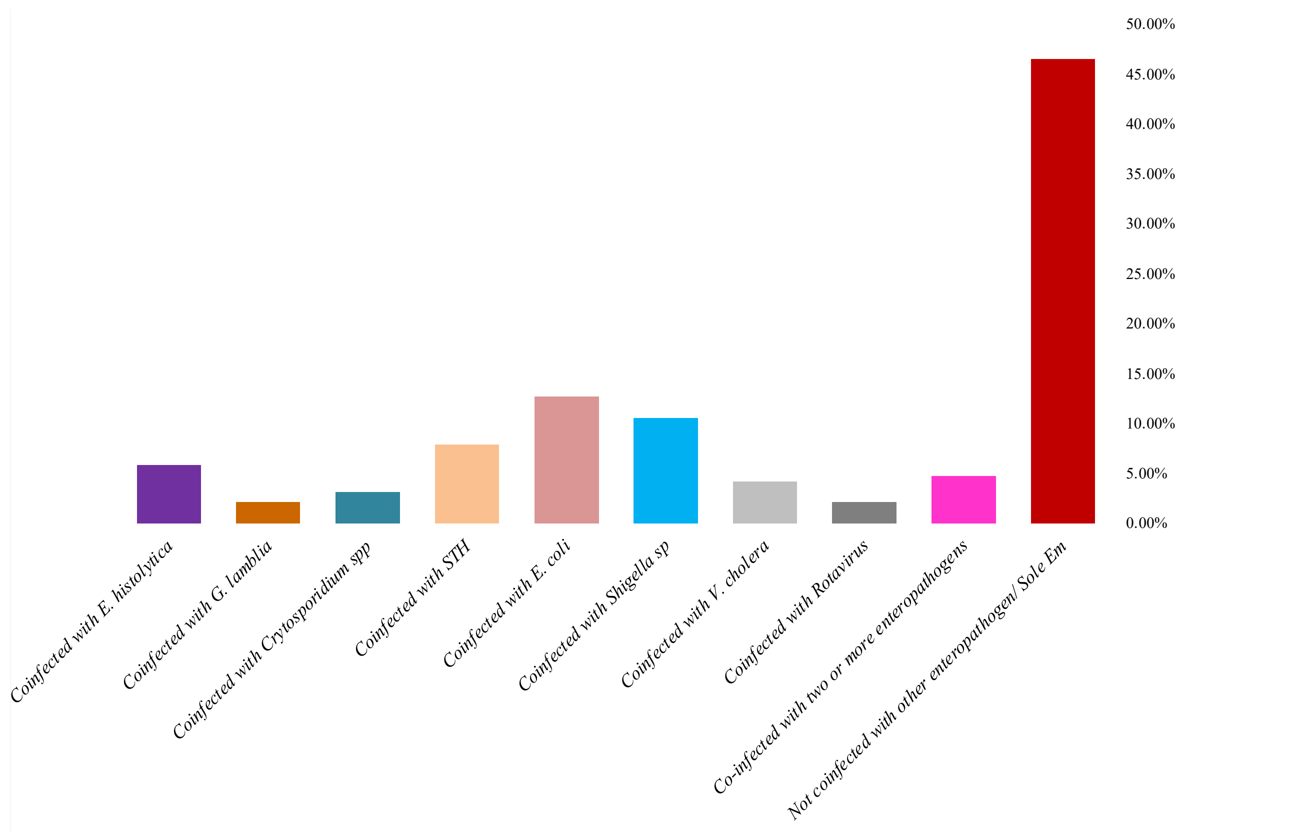


Fig 5.1 Additional enteropathogens in *E. moshkovskii* infected patients. Coinfection with *E. coli* is highest followed by *Shigella* sp and Soil Transmitted helminths (STH).

5.3.2. Seasonal distribution of *Entamoeba moshkovskii* infection

In this three-year study (March 2017 to February 2020), a specific seasonal distribution was found for *Entamoeba moshkovskii* infection. It was observed that the prevalence of *Entamoeba moshkovskii* parasites mostly increased during the post-fall (Sep-Nov) season and in the summer season (April-June) of each year. Moreover, the prevalence of *E. moshkovskii* was notably higher than that *E. histolytica* throughout the three years. Further research over multiple consecutive years is advisable to validate the unique seasonality of *E. moshkovskii*, as higher standard deviation values suggest the need for additional studies (**Fig 5.2**).

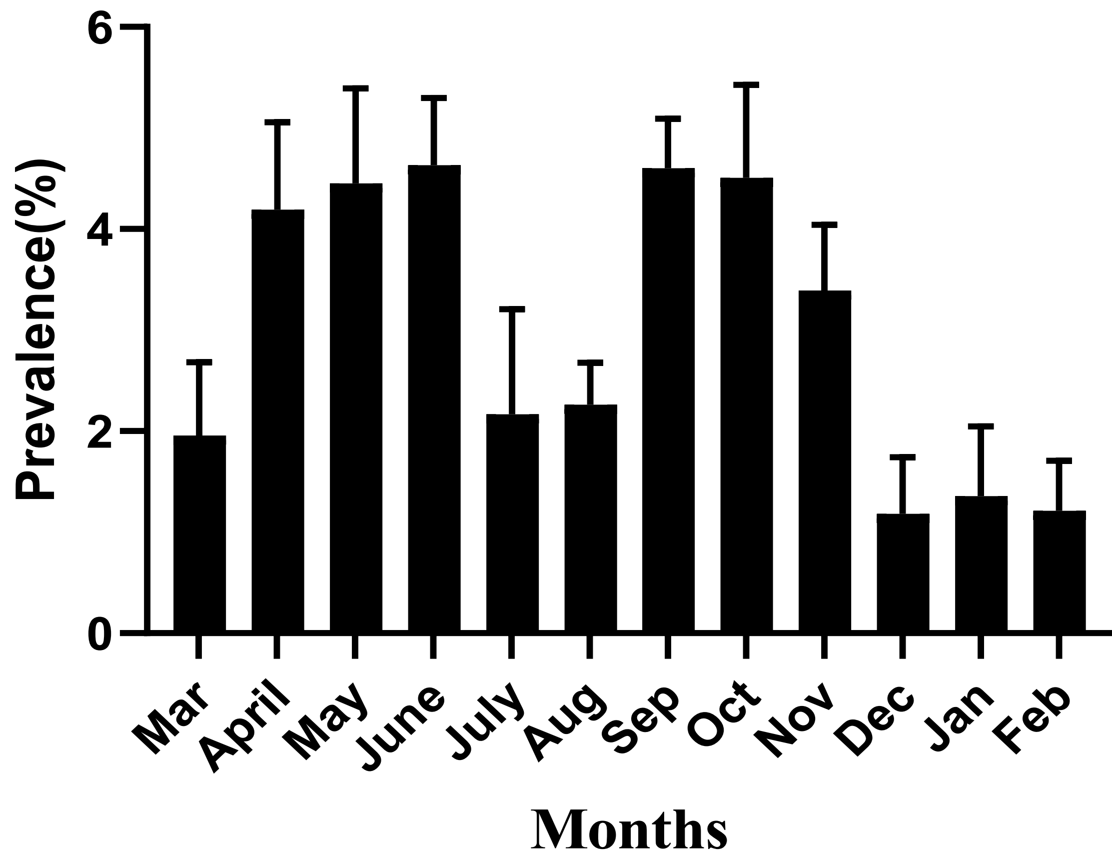


Fig 5.2. Seasonal Distribution of *Entamoeba moshkovskii* in Kolkata and Adjacent Areas from March 2017 to February 2020. The graph illustrates the mean values of *Entamoeba moshkovskii* occurrences over a three-year period, capturing the seasonal distribution in Kolkata and its adjacent areas. Data collected from March 2017 to February 2020 was used to plot the graph, providing insights into the prevalence of Entamoeba moshkovskii throughout different seasons in the region.

5.3.3. *E. moshkovskii* prevalent areas

The prevalence study carried out in the I.D. hospital and B C Roy Children Hospital pointed out that the following areas i.e., Beliaghata, Entally, Kashipur-Belgachia, Maniktala, Gopalpur-Rajarhat, Kolkata-Port, Jorasanko, Baruipurpurba, Maheshtala, Metiabruz, Bhangar around Kolkata were very highly burdened (>3.5%) with *Entamoeba moshkovskii* infection. Ballygunge, Chowringhee, Dumdum Uttar, Dumdum, Bidhannagar, Bhabanipur and Kashba had high prevalence rates (3%-3.5%). Behala Paschim, Behala Purba, Jadavpur were moderately (2.5%-2.99%) infected, while the lowest prevalence (< 2.49%) was reported in Baranagar, Rajarhat New Town and Shyampukur areas (**Fig 5.3**).

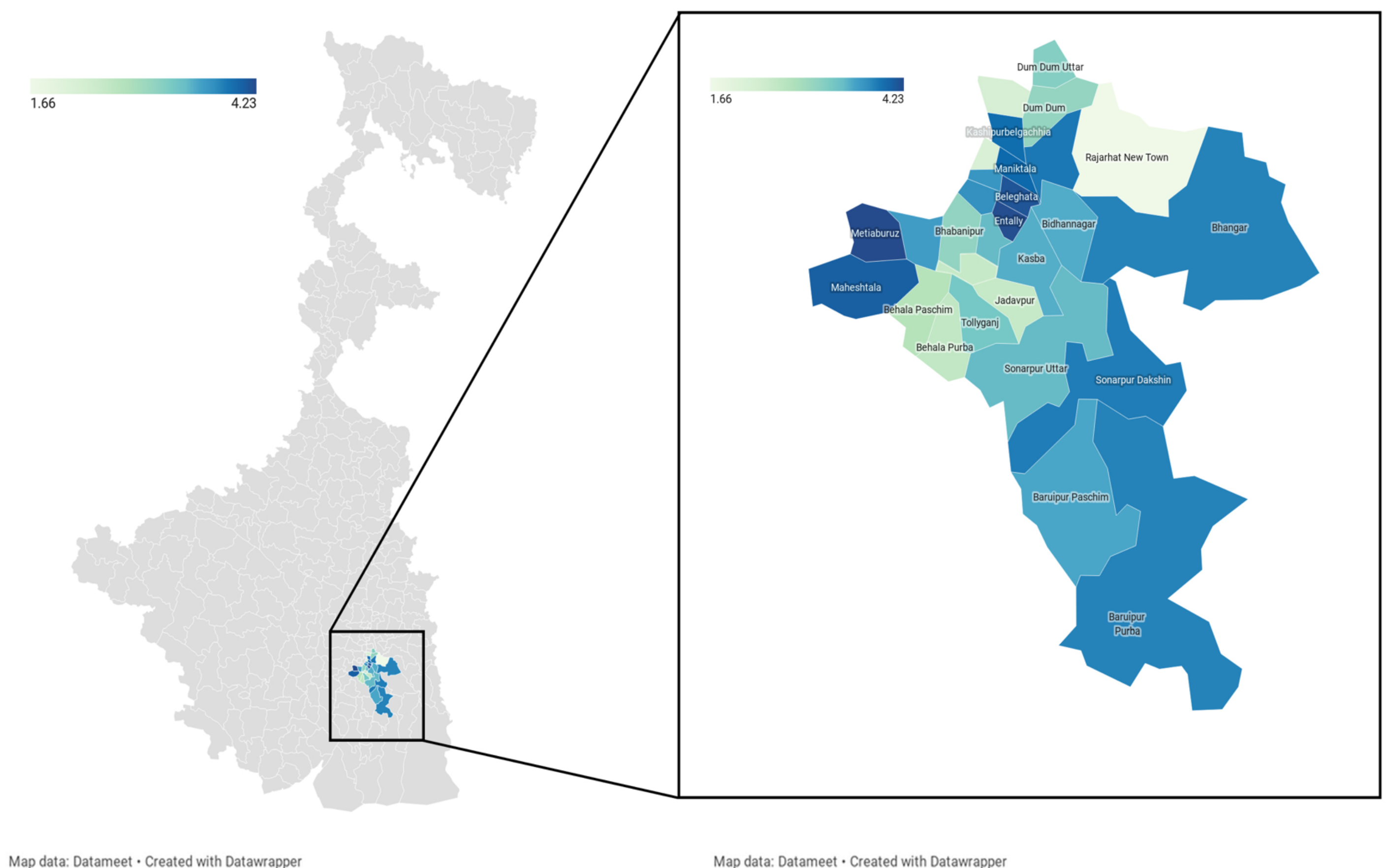


Fig. 5.3 GIS mapping of *E. moshkovskii* infection in and around Kolkata, West Bengal, India.

5.3.4. Phylogenetic and sequencing analysis of local isolates of *E. moshkovskii*

To consider the genetic diversity in *E. moshkovskii* isolates in and around Kolkata, the 18S rRNA gene locus in 68 positive cases was successfully sequenced. Sequencing revealed that the locally found *E. moshkovskii* strains were 99.59% - 100% identical with the prototype (GenBank accession no KP722605.1), and all isolates were the same species. All 68 sequences were deposited in NCBI GenBank under accession numbers ON965383- ON965450 (**Table 5.5**). The nucleotide sequence of *E. moshkovskii* of 36 isolates had 100% genetic identity with the reference sequences (prototype) previously published in GenBank (accession numberAF149906.1), and the rest of the 32 sequences represented genetic variants of *E. moshkovskii* not described earlier. We selected five known sequences, viz. *E. histolytica* (Genbank: AP023147.1), *E. nutalli* (Genbank: LC042219.2), *E. invadens* (Genbank: AF149905.1), *E. chattoni* (Genbank: AF1499121.1), *E. coli* (Genbank: AF149914.1), *Dictyostelium discoideum* (Genbank: AM168039.1), *E. polecki* (Genbank: AF14913.1) to construct the phylogenetic tree of the *E. moshkovskii* isolates obtained in this study. Phylogenetic analysis showed that the study isolates of *E. moshkovskii* formed a monophyletic sister clade to the common ancestors of the *E. histolytica* and *E. dispar* 18S rRNA sequences (**Fig 5.4**). The clades were supported with high bootstrap values. The studied *E. moshkovskii* isolates were grouped with both prototypes (GenBank accession no: AF149906.1) and their close variants. Most of the variants showing the bootstrap value of 89%–99% shared a common clade with the prototype cluster, consisting of two subgroups: sole infection and mixed infection. Some isolates from the mixed infection subgroup are also clustered into separate distant clades from the prototype lineage. The EM IND/37 (GenBank: ON9654419) and EM IND/46 (GenBank: ON9654428) formed a separate group with 100% bootstrap value from the prototypes, which was considered unique due to the presence of one insertion (nucleotide A) and one deletion (nucleotide T) at the studied locus of the isolates (**Fig. 5.4**). This group requires further investigation to gain a better understanding of the evolutionary processes that have shaped them.

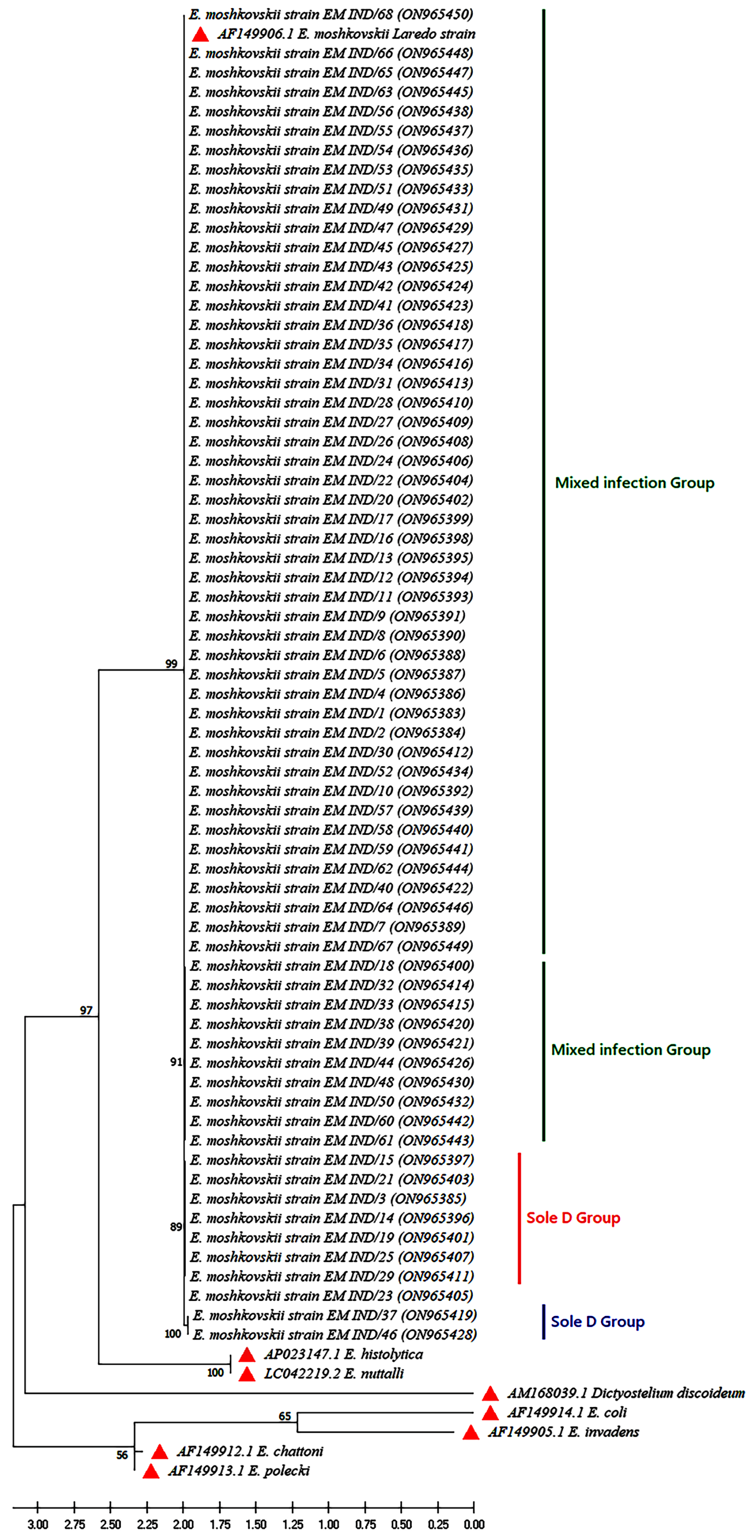


Fig. 5.4 Phylogenetic analysis of study isolates based on 18S rRNA locus of *Entamoeba moshkovskii.*

5.3.5. Overall distribution of SNPs in 18S rRNA locus of *E. moshkovskii*

The sequence of 18r RNA locus of 68 isolates was successfully acquired from positive stool samples. This analysis involved all of the 68 nucleotide sequences. A total of 733 positions were analysed to identify SNPs in the final dataset. Through the sequence of 18S rRNA locus, we have identified 10 SNPs, one deletion and one insertion in 733 sequenced bases from 68 isolates of *E. moshkovskii* (**Table 5.3**). The isolates were 99.60%- 100% identical (GenBank accession no AF149906.1), and all isolates were of the same species (**Table 5.5**). Out of 68 isolates, 36 had sequences identical to the corresponding reference sequence and the rest of the 30 differed by one to three single-nucleotide polymorphisms (SNPs). Two samples had one insertion at position 795 - 796 (nucleotide A) and one deletion at position 769 (Nucleotide T) (refer **Table 5.4** for substitution matrix). All SNPs identified corresponded to transition (pyrimidine <=> pyrimidine/ purine <=> purine) or transversion (purine <=> pyrimidine) mutations. The studied locus of the isolates transition and transversion base substitutions showed the same propensity (50%). The estimated Transition/Transversion bias (*R*) was 0.4. The average base composition in the studied locus was A = 33.57%, T/U = 25.30%, C = 16.80%, and G = 24.33%, with slightly high AT richness in the sequences. The maximum Log-likelihood for this computation was -1181.283. The maximum likelihood estimate of the substitution matrix is presented in Table 5.4. We have also obtained a sum of 28 polymorphic/variable sites, out of which there were 0 singleton variables and 28 were parsimony-informative sites (**Table 5.6**). The parsimony-informative sites gave information about the evolutionary relationships among the isolates. Among 10 SNPs observed in the 18S rRNA locus, five SNPs were potentially associated with specific coinfection incidence. For example, 722 T/C (p= <0.0001) transition was found to be associated with *Entamoeba histolytica* co-infection (IEH). 814 T/G transversion (p= 0.0142) and 826 T/A transversion (p= 0.0142) also exhibited a strong association with diarrhoea-causing bacterial or viral co-infection (IB/V). Only 1345 T/G (p= 0.0424) and 1361 A/G (p= 0.0424) showed a positive correlation with the sole infection of *E. moshkovskii.* However, the deletion of nucleotide A (adenine) at 769 and insertion of nucleotide T (thymine) at 795-796 were not significantly associated with any co-infection incidence. Detailed information on SNPs identified within the target loci of study isolates is provided in **Table 5.5.**

Table 5.3 SNPs/ insertion-deletion identified in 18S rRNA of *E. moshkovskii* study isolates.

SNPs	P VALUE	X^2, df	Significance	Associated group
722 T/C	<0.0001	34.24, 4	Yes	IEH
788 T/C	0.864	1.286, 4	No	–
814 T/G	0.0142	12.46, 4	YES	IBV
826 T/A	0.0142	12.46, 4	YES	IBV
988 G/C	0.293	4.945, 4	No	–
1145G/A	0.1909	6.113, 4	No	–
1345 T/G	0.0424	9.884, 4	Yes	Sole D
1361 A/G	0.0424	9.884, 4	Yes	Sole D
1377 T/G	0.1448	6.836, 4	No	–
1437 G/A	0.2445	5.446, 4	No	–
769 A delete	0.3295	4.612, 4	No	–
795-796 T insert	0.3295	4.612, 4	No	–

Sole D: diarrheal patients solely infected with *E. moshkovskii*, IEH: *E moshkovskii* positive samples co-infected with *Entamoeba histolytica*, IOEP: *E moshkovskii* positive samples co-infected with other Enteric Parasites- *G. lamblia*, *Cryptosporidium* spp, ISTH*: E moshkovskii* positive samples co-infected with soil-transmitted helminths, IB/V: *E moshkovskii* positive samples co-infected with other diarrhoea-causing bacteria-*E. coli*, *Shigella* spp & *V. cholera* or virus-Rotavirus. , P: Correlation coefficient value of the particular association, df: degree of freedom, X^2: Chi-square value.

Table 5.4 Substitution matrix based on 18S rRNA sequences of the populations of *E. moshkovskii* collected in and around Kolkata.

	A	**T/U**	**C**	**G**
A	-	*5.33*	*4.10*	**7.63**
T/U	*7.02*	-	**16.83**	*5.50*
C	*7.02*	**21.89**	-	*5.50*
G	**9.74**	*5.33*	*4.10*	-

Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+I). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 45% sites). Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. Relative values of instantaneous *r* should be considered when evaluating them. For estimating ML values, a tree topology was automatically computed. This evolutionary analysis was conducted in MEGA X.

Table 5.5 List of clinical isolates of *E. moshkovskii* in diarrheal patients obtained from the hospital-based surveillance study.

Sl No.	Name of organism	Id no	Target locus	GenBank Submission Id	Percentage of variation with respect to Prototype (AF149906.1)	SNPs Position	Nature of infection
1	*E. moshkovskii*	EM_IND/1	18S rRNA	ON965383	0%	NA	Sole D
2	*E. moshkovskii*	EM_IND/2	18S rRNA	ON965384	0%	NA	Sole D
3	*E. moshkovskii*	EM_IND/3	18S rRNA	ON965385	0.41%	1345 T/G, 1361 A/G, 1377 T/G	Sole D
4	*E. moshkovskii*	EM_IND/4	18S rRNA	ON965386	0%	NA	Sole D
5	*E. moshkovskii*	EM_IND/5	18S rRNA	ON965387	0%	NA	Sole D
6	*E. moshkovskii*	EM_IND/6	18S rRNA	ON965388	0%	NA	Sole D
7	*E. moshkovskii*	EM_IND/7	18S rRNA	ON965389	0.14%	988 G/C	Sole D
8	*E. moshkovskii*	EM_IND/8	18S rRNA	ON965390	0%	NA	Sole D
9	*E. moshkovskii*	EM_IND/9	18S rRNA	ON965391	0%	NA	Sole D
10	*E. moshkovskii*	EM_IND/10	18S rRNA	ON965391	0.14%	722 T/C	Sole D
11	*E. moshkovskii*	EM_IND/11	18S rRNA	ON965392	0%	NA	Sole D
12	*E. moshkovskii*	EM_IND/12	18S rRNA	ON965393	0%	NA	Sole D
13	*E. moshkovskii*	EM_IND/13	18S rRNA	ON965394	0%	NA	Sole D
14	*E. moshkovskii*	EM_IND/14	18S rRNA	ON965395	0.41%	1345 T/G, 1361 A/G, 1377 T/G	Sole D
15	*E. moshkovskii*	EM_IND/15	18S rRNA	ON965396	0.27%	1345 T/G, 1361 A/G	Sole D
16	*E. moshkovskii*	EM_IND/16	18S rRNA	ON965397	0%	NA	Sole D
17	*E. moshkovskii*	EM_IND/17	18S rRNA	ON965398	0%	NA	
18	*E. moshkovskii*	EM_IND/18	18S rRNA	ON965399	0.27%	814 T/G,	Sole D

						826 T/A	
19	*E. moshkovskii*	EM_IND/19	18S rRNA	ON965401	0.41%	1345 T/G, 1361 A/G, 1377 T/G	Sole D
20	*E. moshkovskii*	EM_IND/20	18S rRNA	ON965402	0%	NA	Sole D
21	*E. moshkovskii*	EM_IND/21	18S rRNA	ON965403	0.27%	1345 T/G, 1361 A/G	Sole D
22	*E. moshkovskii*	EM_IND/22	18S rRNA	ON965404	0%	NA	Sole D
23	*E. moshkovskii*	EM_IND/23	18S rRNA	ON965405	0.14%	788 T/C	Sole D
24	*E. moshkovskii*	EM_IND/24	18S rRNA	ON965406	0%	NA	Sole D
25	*E. moshkovskii*	EM_IND/25	18S rRNA	ON965407	0.41%	1345 T/G, 1361 A/G, 1377 T/G	Sole D
26	*E. moshkovskii*	EM_IND/26	18S rRNA	ON965408	0%	NA	Sole D
27	*E. moshkovskii*	EM_IND/27	18S rRNA	ON965409	0%	NA	Sole D
28	*E. moshkovskii*	EM_IND/28	18S rRNA	ON965410	0%	NA	Sole D
29	*E. moshkovskii*	EM_IND/29	18S rRNA	ON965411	0.41%	1345 T/G, 1361 A/G, 1377 T/G	Sole D
30	*E. moshkovskii*	EM_IND/52	18S rRNA	ON965412	0.14%	1145 G/A	SOLE D
31	*E. moshkovskii*	EM_IND/31	18S rRNA	ON965413	0%	NA	IB/V
32	*E. moshkovskii*	EM_IND/32	18S rRNA	ON965414	0.27%	814 T/G 826 T/A	IB/V
33	*E. moshkovskii*	EM_IND/33	18S rRNA	ON965415	0.27%	814 T/G 826 T/A	IB/V
34	*E. moshkovskii*	EM_IND/34	18S rRNA	ON965416	0%	NA	IB/V
35	*E. moshkovskii*	EM_IND/35	18S rRNA	ON965417	0%	NA	IB/V
36	*E. moshkovskii*	EM_IND/36	18S rRNA	ON965418	0%	NA	IB/V
37	*E. moshkovskii*	EM_IND/37	18S rRNA	ON965419	0.27%	769 A delete	IB/V

						795-796 T insert	
38	*E. moshkovskii*	EM_IND/38	18S rRNA	ON965420	0.27%	814 T/G 826 T/A	IB/V
39	*E. moshkovskii*	EM_IND/39	18S rRNA	ON965421	0.27%	814 T/G 826 T/A	IB/V
40	*E. moshkovskii*	EM_IND/40	18S rRNA	ON965422	0.14%	1437 G/A	IB/V
41	*E. moshkovskii*	EM_IND/41	18S rRNA	ON965423	0%	NA	IB/V
42	*E. moshkovskii*	EM_IND/42	18S rRNA	ON965424	0%	NA	IB/V
43	*E. moshkovskii*	EM_IND/43	18S rRNA	ON965425	0%	NA	IB/V
44	*E. moshkovskii*	EM_IND/44	18S rRNA	ON965426	0.27%	814 T/G 826 T/A	IB/V
45	*E. moshkovskii*	EM_IND/45	18S rRNA	ON965426	0%	NA	IB/V
46	*E. moshkovskii*	EM_IND/46	18S rRNA	ON965427	0.27%	769 A delete, 795-796 T insert	IB/V
47	*E. moshkovskii*	EM_IND/47	18S rRNA	ON965428	0%	NA	IB/V
48	*E. moshkovskii*	EM_IND/48	18S rRNA	ON965429	0.27%	814 T/G 826 T/A	IB/V
49	*E. moshkovskii*	EM_IND/49	18S rRNA	ON965430	0%	NA	IB/V
50	*E. moshkovskii*	EM_IND/50	18S rRNA	ON965431	0.27%	814 T/G 826 T/A	IB/V
51	*E. moshkovskii*	EM_IND/51	18S rRNA	ON965432		NA	IB/V
52	*E. moshkovskii*	EM_IND/52	18S rRNA	ON965433	0.14%	1145 G/A	ISTH
53	*E. moshkovskii*	EM_IND/53	18S rRNA	ON965434	0%	NA	ISTH
54	*E. moshkovskii*	EM_IND/54	18S rRNA	ON965435	0%	NA	ISTH
55	*E. moshkovskii*	EM_IND/55	18S rRNA	ON965436	0%	NA	ISTH

56	*E. moshkovskii*	EM_IND/56	18S rRNA	ON965437	0%	NA	ISTH
57	*E. moshkovskii*	EM_IND/57	18S rRNA	ON965438	0.14%	722 T/C	IEH
58	*E. moshkovskii*	EM_IND/58	18S rRNA	ON965439	0.14%	722 T/C	IEH
59	*E. moshkovskii*	EM_IND/59	18S rRNA	ON965440	0.14%	722 T/C	IEH
60	*E. moshkovskii*	EM_IND/60	18S rRNA	ON965441	0.27%	814 T/G 826 T/A	IEH
61	*E. moshkovskii*	EM_IND/61	18S rRNA	ON965442	0.27%	814 T/G 826 T/A	IEH
62	*E. moshkovskii*	EM_IND/62	18S rRNA	ON965443	0.14%	722 T/C	IEH
63	*E. moshkovskii*	EM_IND/63	18S rRNA	ON965445	0%	NA	IOEP
64	*E. moshkovskii*	EM_IND/ 64	18S rRNA	ON965446	0.14%	1437 G/A	IOEP
65	*E. moshkovskii*	EM_IND/65	18S rRNA	ON965447	0%	NA	IOEP
66	*E. moshkovskii*	EM_IND/66	18S rRNA	ON965448	0%	NA	IOEP
67	*E. moshkovskii*	EM_IND/67	18S rRNA	ON965449	0.14%	988 G/C	IOEP
68	*E. moshkovskii*	EM_IND/68	18S rRNA	ON965450	0%	NA	IOEP

Sole D : diarrheal patients solely infected with *E. moshkovskii*, IEH: *E moshkovskii* positive samples co-infected with *Entamoeba histolytica*, IOEP: *E moshkovskii* positive samples co-infected with other Enteric Parasites- *G. lamblia*, *Cryptosporidium* spp, ISTH *: E moshkovskii* positive samples co-infected with soil transmitted helminths, IB/V: *E moshkovskii* positive samples co-infected with other diarrhea causing bacteria-*E. coli*, *Shigella* spp & *V. cholera* or virus-Rotavirus.

5.3.6. Median-Joining haplotype network

In total, 68 sequences of the 18S rRNA locus were used to assess the relationship of *E. moshkovskii* haplotypes among different coinfected subgroups. Ten distinct haplotypes were identified in this study. Haplotype 1 was widespread, occurring in both coinfected subgroups viz. IOEP, ISTH and IB/V and the sole *E. moshkovskii*-infected subgroup (Sole D). The stellate shape of the constructed network suggests a rapid expansion of the population of *E. moshkovskii* in the study region (**Fig 5.5**).

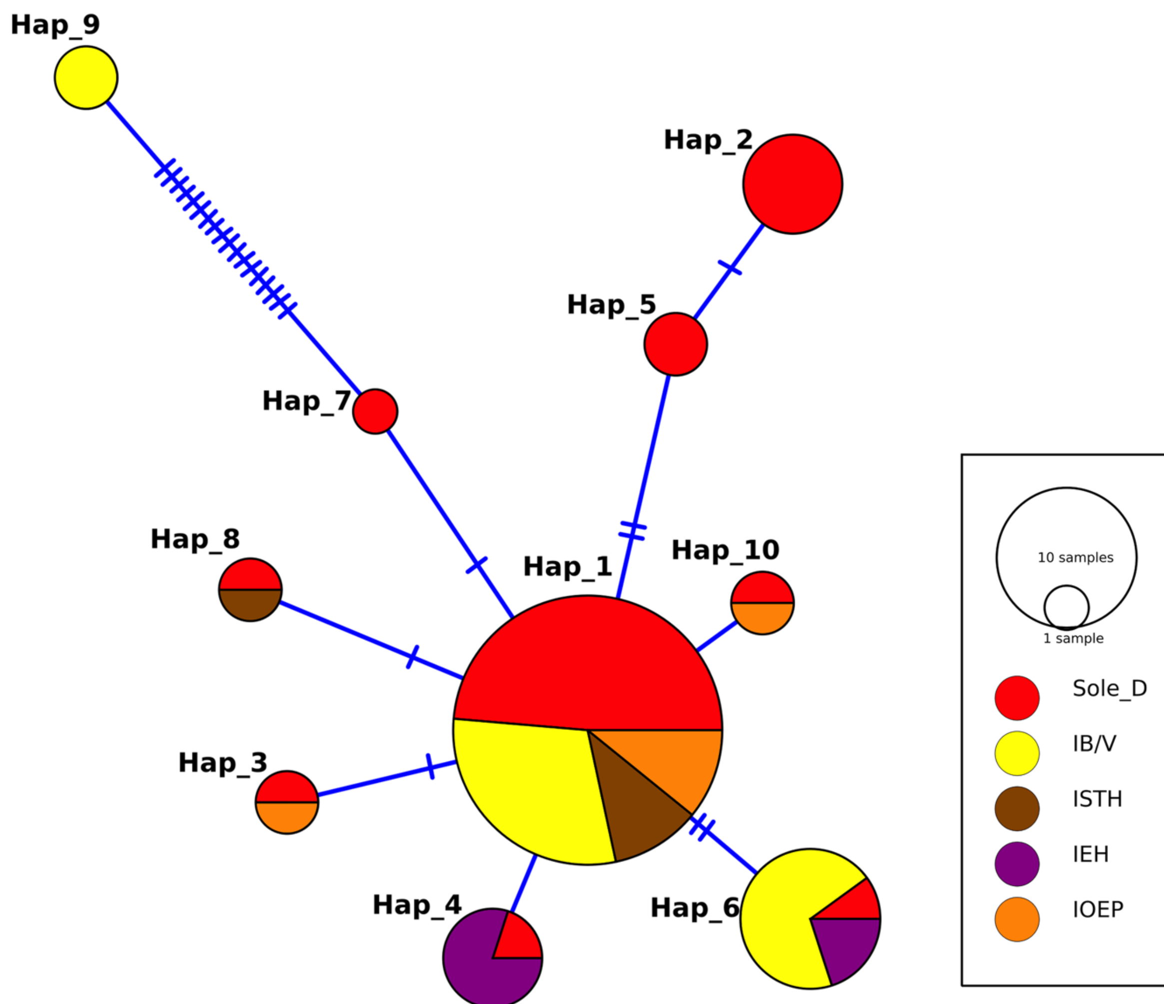


Fig. 5.5 Median-joining network of 68 *E. moshkovskii* haplotypes obtained in and around Kolkata, West Bengal, India. Sole D: diarrheal patients solely infected with E. moshkovskii, IEH: *E. moshkovskii* positive samples co-infected with *E. histolytica*, IOEP: *E. moshkovskii* positive samples co-infected with other Enteric Parasites- G. lamblia, Cryptosporidium spp, ISTH: *E. moshkovskii* positive samples co-infected with soil-transmitted helminths, IB/V: *E. moshkovskii* positive samples co-infected with other diarrhoea-causing bacteria-*E. coli, Shigella* spp &*V. cholera* or virus-Rotavirus.

5.3.7. Population structure:

5.3.7.1. *Genetic diversity:*

We have investigated the following descriptive statistics of genetic diversity using the software DnaSP v5: number of segregating sites (S), number of haplotypes (*h*), haplotype diversity (HD), and nucleotide diversity (π) and Average number of nucleotide (K). Genetic diversity indices revealed a total of 28 segregating sites (S) and 10 haplotypes (*h*) (**Fig 5.6**), as well as haplotype diversity (HD) of 0.677 ±0.057 and nucleotide diversity (π) of 0.00336 ±0.00100. We also investigated the same for different co-infected/sole *E. moshkovskii* infected subgroups. The values are provided in **Table 5.6.** The haplotype diversity (HD) for different subgroups ranges from 0.400 ±0.237 to 0.633 ±0.074, and the nucleotide diversity (π) ranges from 0.00055 ±0.00032 to 0.00609 ±0.00266 across all sites. The average number of Nucleotide differences (*k*) for each subgroup ranged from 0.40000 to 4.4667. The study found a high degree of haplotype diversity in the Sole D subgroup and low nucleotide diversity, except for those co-infected with Soil-transmitted helminths (ISTH) which showed lower haplotype diversity. The Sole D subgroup had the most haplotypes (8 haplotypes), with a high HD value. Hence the apparent observation from this study is that there is a high level of genetic diversity in the Sole D subgroup compared to other subgroups. The Sole D subgroup had two separate subclades in the phylogenetic tree, the first containing Haplotype_2 and Haplotype_5 with a bootstrap value of 91, and the second containing only Haplotype_9. But no clustering was seen in the phylogenetic tree for other co-infected subgroups, and there was no geographical or seasonal pattern. Most novel haplotypes were found to co-occur with the most prevalent haplotype. Haplotypes Hap_2, Hap_7, and Hap_5 were only found in the Sole *E. moshkovskii* infected subgroup, with Hap_2 appearing exclusively in the subgroup co-infected with IB/V.

Three neutrality tests (Tajima' D, Fu & Li's D and Fu & Li's F value) showed negative non-significant values for Sole D, ISTH and IOEP subpopulations (**Table 5.6**). These analyses indicated deviations from neutrality and implied population expansion (e.g., after a bottleneck event or a selective sweep) and/or purifying selection in the three infected subgroups of *E. moshkovskii*. The negative Tajima' D value of the IB/V subgroup (Tajima' D= -1.02116) also suggested that this population deviated from the standard neutral. The three neutrality tests for the IEH subgroup resulted in positive values spectacled balancing selection or sudden population contraction. However, statistically non-significant value revealed a weak selection within and among all study sites. The estimation of another neutrality test, Fu's F_S based on

the haplotype distribution, showed negative values for Sole D and IOEP subpopulations, demonstrating an excess of rare haplotypes compared to what would be expected under neutrality. Sole D exhibited the lowest Fu's F_S value (Fu's *Fs*= -2.506) which conferred the hypothesis of past population expansion incidents for the subgroup. However, positive values of Fu's F_S in IEH, ISTH and IB/V subgroups suggested population subdivisions and overdominant selection or bottlenecks. IB/V subgroups showed the highest degree of positive Fu's F_S value (Fu's *Fs*= 5.167) due to the presence of haplotype (Hap_9) with insertion–deletion polymorphisms. Strong positive neutrality results for IEH (Fu's Fs= 2.506*;* Tajima' D= 1.12414; Fu & Li's D= 1.39584 and Fu & Li's F= 1.40624) subgroups highly supported a sudden population contraction and/or balancing selection (**Table 5.6**).

Tajima' D results in overall negative values (Tajima' D= -1.83320; $p<0.05$) from both the tests that there is an excess of rare mutations in the subgroups, and the excess is statistically significant. The overall negative values (Fu's F_S = -0.544) also resulted from Fu's F_S test. However, the overall Fu & Li's D and Fu & Li's F (Fu & Li's D= 1.85839, $p<0.02$; Fu & Li's F= 0.58884) values were positive, and this revealed an excess of ancestral/prototype variants, that have been selected for in the past (**Table 5.6 and Fig 5.7**). In other words, the number of unique variants present was low, and the ones that were present were carried by a large number of individuals.

Pairwise fixation index (F_{ST}) values among the different coinfected groups were assessed for measuring population differentiation based on their level of genetic differentiation. Pair-wise F_{ST} values were also obtained from the comparison between specific co-infected/sole-infected subgroups, and these values were assessed to measure population differentiation. An F_{ST} greater than 0.15 can be interpreted as very little gene flow and is significant in differentiating populations (Ajogbasile et al. 2021). According to Table 3 shown, very little gene flow was obtained among the subgroups of Sole D with IEH, IOEP with IEH and the subgroups of IEH with ISTH. Consequently, very high genetic differentiation was observed in these subgroups. The highest F_{ST} value was estimated between co-infected subgroup IEH against subgroups ISTH (F_{ST} value = 0.34783) in all possible combinations of co-infected/Sole infected subgroups. The co-infected subgroups IOEP against ISTH exhibited the lowest F_{ST} value (F_{ST} value = 0.0000) (**Table 5.7**), indicating the highest level of gene flows between these two subpopulations with the shortest distance and the most increased accessibility. Overall the estimated genetic differentiation index was highly statistically significant (X^2=77.195, $P<0.001$) among the parasite subpopulations.

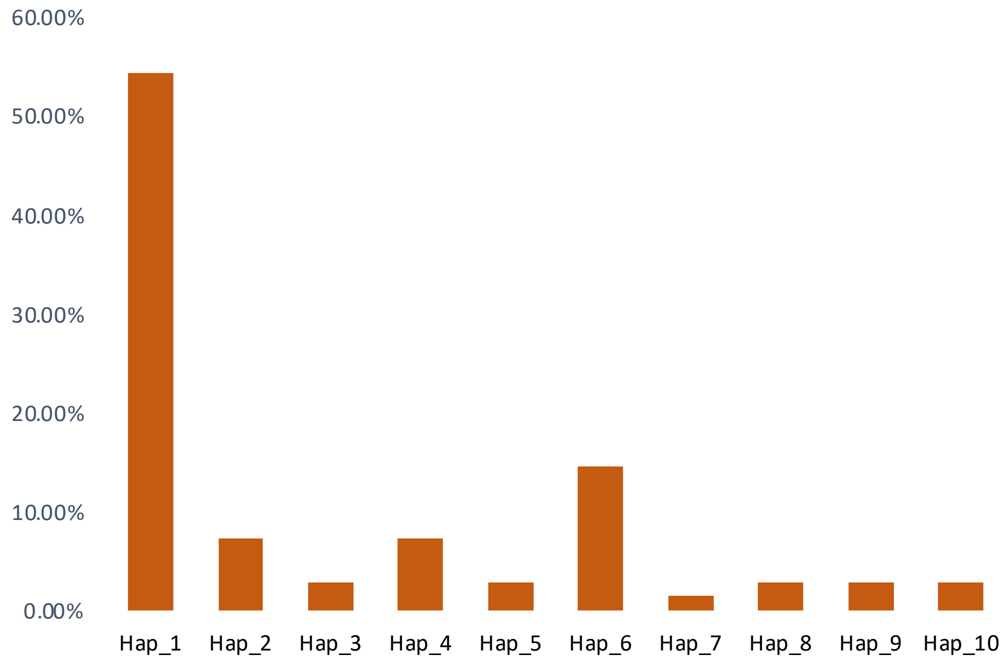


Fig 5.6 Haplotype distribution *E. moshkovskii* isolates obtained in the study area. Hap_1 represents the sequence of the Laredo strain of *E. moshkovskii*.

Table 5.6 Genetic diversity indices and neutrality tests based on 18SrRNA sequences found in local isolates of *E. moshkovskii* in Kolkata and adjacent areas. Haplotype_1(Prototypes) was identical to Laredo strain of *E. moshkovskii*.

Subgroups	N	Haplotypes obtained	S	h	K	Hd +/-SD	Π +/-SD	**Fu's *Fs***	**Tajima's D**	**Fu & Li's *D***	**Fu & Li's *F***
Sole D	30	Hap_1, Hap_2, Hap_3, Hap_4, Hap_5, Hap_6, Hap_7, Hap_8	9	8	1.42759	0.623+/-0.093	0.00195+/0.00036	-2.506	-1.15479	-2.12379	-2.13591
IEH	6	Hap_4, Hap_6	3	2	1.60000	0.533+/-0.172	0.00218+/0.00070	2.506	1.12414	1.39584	1.40624
IOEP	6	Hap_1, Hap_3, Hap_10	2	3	0.66667	0.600+/-0.215	0.00091+/0.00038	-0.858	-1.13197	-1.15529	-1.19511
ISTH	5	Hap_1, Hap_8	1	2	0.40000	0.400+/-0.237	0.00055+/0.00032	0.90	-0.81650	-0.81650	-0.77152
IB/V	21	Hap_1, Hap_6, Hap_9, Hap_10	22	4	4.4667	0.633+/-0.074	0.00609+/0.00266	5.167	-1.02116	1.35707	0.75547
Total	68		28	10	2.46313	0.677+/-0.057	0.00336+/0.00100	-0.544	-1.83320**	1.85839*	0.58884

N: Sample size; S: number of polymorphic/segregating sites; *h*: number of haplotypes; K: Average number of nucleotide differences; Hd: haplotype diversity; : π nucleotide diversity

**Statistically significant, $p<0.05$

*Statistically significant, $p<0.02$

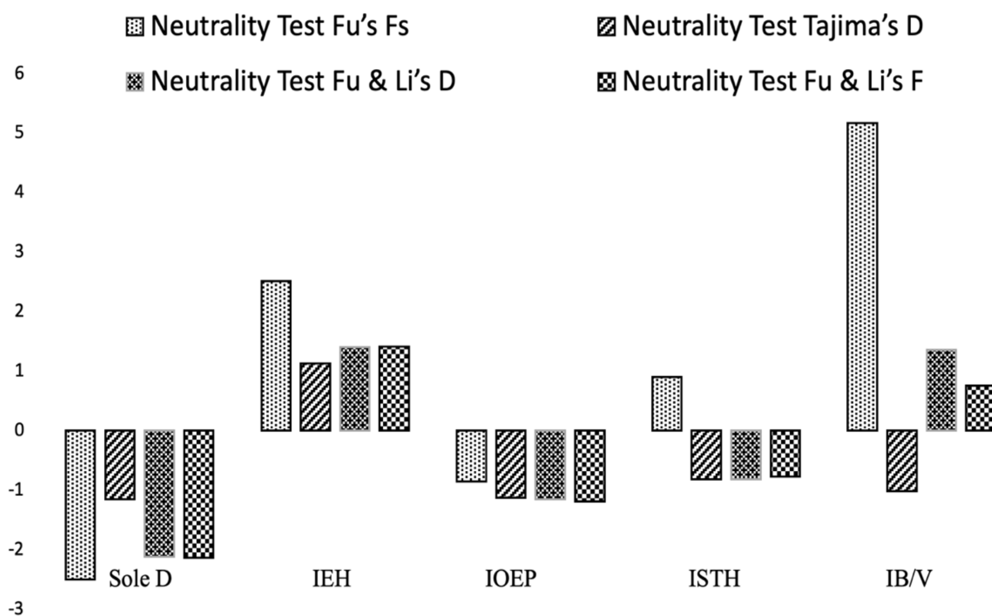


Fig 5.7 Values of different neutrality tests in different coinfected groups of *E. moshkovskii*.

Table 5.7 Genetic differentiation (F_{ST}) among different coinfected subgroups of *Entamoeba moshkovskii*

Sub group	Sole D	IEH	IOEP	ISTH	IB/V
Sole D	0.0000				
IOEP	0.09383	0.32000	0.0000		
ISTH	0.10412	0.34783	0.0000	0.0000	
IB/V	0.10865	0.11116	0.09665	0.10664	0.0000

5.3.8. Animal model study- infection of mice with *E. moshkovskii*

All three mice tested positive for *E. histolytica* infection, resulting in a 100% infection rate. Two out of three mice (66.66%) were found to be infected with *E. moshkovskii*. Among the mice infected with *E. moshkovskii*, one mouse (33.33%) displayed symptoms of diarrhea. The affected mice exhibited thickened and contracted ceca. Upon conducting Scanning Electron Microscopy (SEM) on the ceca samples from these mice, the presence of *E. moshkovskii* infection was observed in the epithelial layer of the ceca (**Fig 5.8**). Significant weight loss was noted in CBA/J mice throughout the progression of both *E. histolytica* and *E. moshkovskii* infections. These findings suggest that the *E. moshkovskii* isolates possess virulent properties in mice. This observation aligns with a previous study by Shimokawa et al. in 2012, which reported the virulence of the Laredo strain of *E. moshkovskii* in mice.

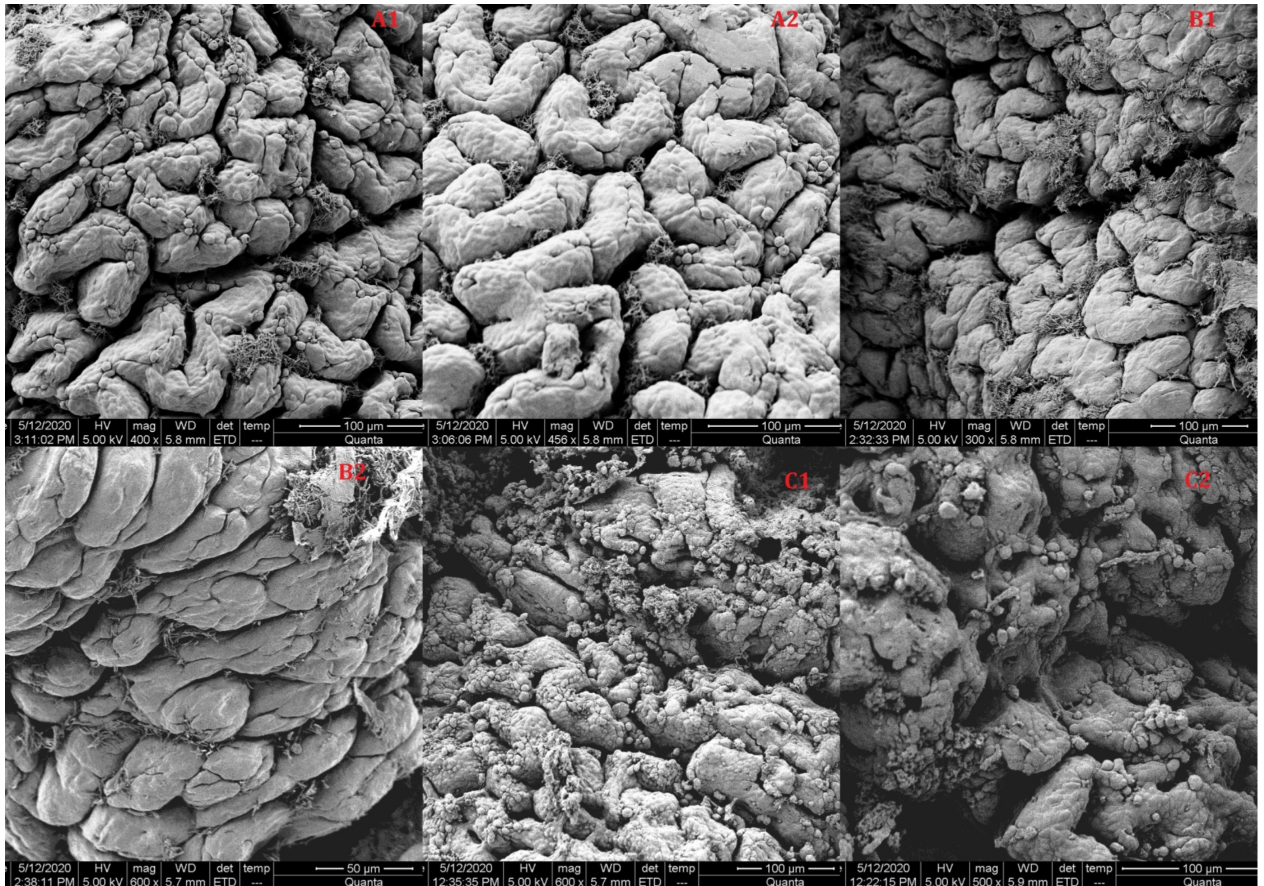


Fig 5.8 Scanning Electron Microscopy Observations. A1 & A2: Scanning electron microscopy images showing the observations of the ceca of CBA/J mice that were successfully infected with *E. moshkovskii*. B1 & B2: Scanning electron microscopy images showing the observations of the ceca of CBA/J mice that were not infected with any *Entamoeba* species. C1 & C2: Scanning electron microscopy images showing the observations of the ceca of CBA/J mice that were successfully infected with *E. histolytica*.

5.4. Discussion

According to the active surveillance study for the detection of common enteric parasites going on for the last two decades, we have observed a decreasing trend of *E. histolytica* infection in the last few years around Kolkata (Mukherjee et al. 2009; Karmakar et al. 2017; Mukherjee et al. 2010). Also, non-seasonal sporadic infections with *E. histolytica* have been observed, which is unusual for a tropical area like Kolkata (Mukherjee et al. 2009; Mukherjee et al. 2010). Microscopic investigation of diarrheal stool samples has uncovered the presence of cysts/trophozoites of amoeba that have a similar morphological feature to *E. histolytica* by a significant proportion throughout the year in Kolkata. After performing PCR-based molecular identification in the previous chapter, it was found that the most abundant species of *Entamoeba* observed in diarrheal stool samples in Kolkata is a morphologically indistinguishable amoeba from *Entamoeba histolytica* and is a related species called *Entamoeba moshkovskii*. A recent study in Egypt showed that 85% of amoebic infections were caused by so called non-pathogenic *Entamoeba* spp. such as *E. dispar*, *E. moshkovskii*, *and E. hartmani* (Abozahra et al. 2020). This observation has led to an increased interest in the study of traditionally non-pathogenic *Entamoeba* species. Humans are a true host for amphizoic amoeba *E. moshkovskii* (Shimokawa et al. 2012). Moreover, recent evidence from different studies supports the pathogenicity of *E. moshkovskii* (Ali et al. 3003; Shimokawa et al. 2012; Parija and Khairnar 2005). Although most epidemiological studies have not adequately assessed the endemicity of *E. moshkovskii*, despite its identification as a cause of human infection (Samie et al. 2020). Within this chapter, we utilized 18S rRNA sequences from sequenced *E. moshkovskii* isolates and employed molecular techniques to investigate the genetic and phylogenetic makeup of *E. moshkovskii* in Kolkata and adjacent areas.

Our research findings highlighted the potential pathogenicity of *Entamoeba* spp, along with other enteric pathogens. More than half of the amoebic infection was caused by *Entamoeba moshkovskii*. This finding is alarming as it implies that *E histolytica* infections previously decreased and *E. moshkovskii* has been taking its place. Although *E. moshkovskii* was highly prevalent in diarrheal patients, we did not find any hematophagous trophozoites of *E. moshkovskii* during microscopy, indicating its non-invasive nature. These results will boost research for a better understanding of the mechanism of pathogenicity in the parasite. A recent study in Bangladesh reported that 21% of children aged 2–5 were infected with *E. moshkovskii*, which was associated with diarrhoea (Ali et al. 2003). Many other studies also reported *E.*

moshkovskii as an enteropathogen in patients suffering from diarrhoea or dysentery (Heredia et al. 2012, Ali et al. 2003, Shimokawa et al. 2012, Kyany'a et al. 2019). Our study was conducted among patients with diarrhoeal complaints. A notable percentage of individuals were infected with *E. moshkovskii,* and the presence of mono-infection/ sole infection of *E. moshkovskii* was statistically associated with diarrhoeal occurrence. Therefore, the diarrheal incidents associated with *E. moshkovskii* were not commonly coinfected in Kolkata. These results indicate that *E. moshkovskii* may not simply be a commensal of the human gut; instead, it acts as a "potential" pathogen causing diarrhoea and other gastrointestinal disorders in the study area. Furthermore, in the murine model of intestinal amebiasis, *E. moshkovskii* was found to induce diarrhoea, weight loss, and colitis, indicating its shared pathogenicity with *E. histolytica*. However, the infection rate of *E. moshkovskii* was lower compared to *E. histolytica*. Further comprehensive research is required to uncover the complete pathogenic nature of *E. moshkovskii*.

A typical seasonal pattern generally observed in many parasitic infections like *E. histolytica* and *Giardia lamblia* usually showed the highest peaks in the wet season. It gradually decreased with the arrival of the dry season (Mbae et al. 2013, Calegar et al. 1994, Vargas et al. 2004). Interestingly we observed a unique seasonal pattern of *E. moshkovskii* infection in Kolkata. We reveal two remarkable peaks of infection in summer and post-fall season. The result of such an unusual study finding has yet to be explained, requiring further detailed investigations.

Further, we have performed phylogenetic analysis and multiple alignments of the *E. moshkovskii* population from Kolkata based on the 18S rRNA locus. Multiple alignments showed that 44.12% (n/N= 30/68) isolates were 100% similar at the sequenced region compared to Laredo strain of *E. moshkovskii,* and the rest of the isolates were novel genetic variants. Phylogenetic analysis clarified the relationships among subpopulations clustered together with their respective variants. However, further research is needed using high-resolution molecular markers to conclude whether the subpopulation is a different genotype of *E. moshkovskii* or a completely new lineage. Since this was a hospital-based surveillance study and patients came from a limited geographical area, the distribution of different genotypes needed to be better understood. But the findings of this study confirmed the distribution of a richly diverse population of *E. moshkovskii* species in Kolkata and adjacent areas. The findings of this study highlighted the epidemiological significance of *E. moshkovskii* infection in Eastern India as it is the first report in this geographical area and exposes the existence of this species as a possible emerging enteric pathogen in India.

Most research on *E. moshkovskii* infection has mainly focused on the prevalence of this pathogen without considering the co-occurrence of other enteric pathogens (Ngui et al. 2012). In this study, a correlation was observed between 18S rRNA SNPs and clinical features. However, the correlation between SNPs and clinical features does not necessarily indicate a direct control of their impact on pathogenicity. However, the sequence of the 18S ribosomal RNA can be used as a phylogenetic marker, allowing for the identification of pathogenic organisms in clinical samples. It can also be utilized to detect *E. moshkovskii* isolates and enables further diagnostic testing. The identified SNPs may exhibit an essential role of *E. moshkovskii* in adapting to the gut environment or in acquiring other enteric pathogens. *E. moshkovskii* infection may alter susceptibility to infection with other enteric pathogens and modulate the effects of co-infecting gut pathogens. *E. moshkovskii* may cause inflammation in the gut and create a niche that helps in the survival and proliferation of other enteropathogenic bacteria/viruses. The observed multiple infections with many helminths and protozoan parasites could be explained by their same mode of transmission and poor hygiene areas. Co-infection of *E. moshkovskii* and *E. histolytica* also suggests the absence of competitive exclusion among them. We also did not document any invasive disease in the diarrheal patients infected with *E. moshkovskii.* More interestingly, the *E. moshkovskii* and *E. histolytica* co-infected subgroup also did not show any invasive disease indicating the parallel evolution of these two species. Therefore, the mystery is to be revealed under what conditions *E. moshkovskii* cells turn pathogenic, as many diarrhoeal incidences were observed that were solely infected with this species. This capability may result from particular parasite genetics and/or genotypes in *E. moshkovskii* isolates.

The nucleotide diversity (π) value is an important index in molecular genetics to determine the degree of genetic polymorphism within a population (Mao et al. 2015). The estimator of nucleotide diversity, π, with a higher value than 0.01, suggests comparatively significant variations in most organisms (Mao et al. 2015, Neigel and Avise 1993). In this study, nucleotide diversity for all subgroups was lower than 0.01, indicating a lower degree of genetic polymorphism among the haplotypes. The haplotype diversity index was highest in the IB/V subgroup and lowest in the ISTH subgroup. The average haplotype diversity index was 0.677, considering differences among the haplotypes of each subgroup. The obtained average haplotype diversity index suggests that the *E. moshkovskii* population is highly diverse in this geographical area and influenced by a moderate recombination rate (Mao et al. 2015). The high levels of genetic diversity suggested the strong viability and adaptability of the *E. moshkovskii*

population. This may increase the average fitness of *E. moshkovskii* populations in a changing environment. However, the 18S rRNA data showed that the average nucleotide diversity was reasonably low. The nucleotide diversity was low since it is a highly conserved component with minimal nucleotide substitution rates. Our neutrality test results implied that the Sole D and IOEP subpopulations were not under directional selection pressure. IEH subpopulation was influenced by selection pressure, which resulted in the adaptation of these isolates to coexist with *E. histolytica* via changes in its genetic constitution. However, Pairwise genetic differentiation (F_{ST}) among different coinfected subgroups ranged from low to high. The F_{ST} can range from 0 to 1, where 0 suggests complete sharing of genetic material and 1 suggests no sharing (Mao et al. 2015, Deng et al. 2020). According to the standard F_{ST} scale, the fixation index is F_{ST} less than 0.05 = little genetic difference; F_{ST} of 0.05 0.15 = moderate genetic difference; F_{ST} of 0.15–0.25 = great genetic difference and F_{ST} greater than 0.25 = very great genetic difference (Ajogbasile et al. 2021). The majority F_{ST} values of different coinfected subgroups obtained in this study were lower than 0.25, indicating low to moderate genetic differentiation within the different coinfected/sole infected subgroups of *E. moshkovskii* population in this geographical area. Whereas F_{ST} values with the highest levels of differentiation were observed for IEH subgroups versus three other subgroups– Sole D, IOEP and ISTH are indicating a higher genetic differentiation and higher genetic drift or lower gene flow within IEH coinfected subgroups of the parasite (Deng et al. 2020). The higher F_{ST} values also implied that this genome region may have undergone positive selection pressure in IEH coinfected subgroup (Das et al. 2021). Therefore, the obtained IEH subgroup might be genetically isolated and corresponds to the speciation process. More studies should be performed using other genetic markers to validate whether the coinfected subgroup of *E. moshkovskii* really corresponds to a new lineage or only to a different genotype of *E. moshkovskii*; it should be remembered that we only considered a fragment of 18S rRNA gene in this study.

This chapter presents a comprehensive study of *E. moshkovskii* isolates, focusing on their analysis using the 18S rRNA as a genetic marker. In the next chapter, our study aims to enhance the characterization of the *E. moshkovskii* population by utilizing a range of potential genetic markers. This approach will enable us to identify distinct pathogenic strains solely responsible for causing diarrheal incidents in individuals infected with *E. moshkovskii*.

5.5. Conclusion

In conclusion, the present study suggested that *E. moshkovskii* is one of the causative agents for acute diarrhoea in humans. The study found that many diarrhoeal patients infected with this species were negative for other enteric pathogens such as bacteria and viruses. The study recommends further research to understand the transmission dynamics of *E. moshkovskii* and proper diagnosis to avoid the development of drug-resistant strains. It also highlights the need for public health authorities to implement prevention and control strategies. The findings of the study raise concerns about the importance of proper diagnosis and control of *E. moshkovskii* infection.

CHAPTER VI

GENETIC CHARACTERIZATION OF THE *E. MOSHKOVSKII* POPULATION BASED ON DIFFERENT POTENTIAL GENETIC MARKERS

Genetic characterization of the *E. moshkovskii* population based on different potential genetic markers

6.1. Background

In the previous chapter, the population of *E. moshkovskii* obtained in the specified area was divided into different subgroups according to their co-infection status. The objective of this chapter was to examine the parasite genotypes of the subgroup affected by diarrhoea, which consisted solely of *E. moshkovskii* infections, as well as those co-infected with other pathogens causing diarrhoea. To achieve this, a multi-locus sequence typing (MLST) approach was employed.

In the previous chapter, we found some significant SNPs that were linked to clinical characteristics. However, it's important to note that the correlation between SNPs and clinical features does not necessarily mean that they directly impact pathogenicity. While genotyping using 18S rRNA is essential in phylogenetic analysis, it doesn't directly affect pathogenicity. Therefore, we need to explore alternative approaches to understand how *E. moshkovskii* genotypes control pathogenicity.

Efficient identification and genetic characterization of clinical isolates from endemic areas worldwide play a crucial role in understanding the impact of parasite genomes on amoebic infections. One valuable tool for this purpose is Multilocus Sequence Typing (MLST), which offers a portable, reproducible, and scalable system for typing. Various laboratories can easily perform MLST, as demonstrated by previous studies (Klint et al., 2007; Bom et al., 2011; Xia and Xiong, 2014; de Vries et al., 2015; Urwin and Maiden, 2003). There have been successful developments of several MLST (Multi-Locus Sequence Typing) systems for characterizing strains of *E. histolytica*. To effectively genotype the selection of appropriate genetic markers is vital. In our MLST study, we have integrated three potential genetic markers associated with the diarrhoeal incidence of *E. moshkovskii* infection. These markers include KERP1, a protein rich in lysine and glutamic acid, as well as amoebapore C (apc) and Chitinases. By incorporating these markers into our genotyping system, we aim to enhance the characterization of *E. moshkovskii* strains and gain further insights into their role in diarrhoeal infections.

KERP1 is a protein abundant in lysine and glutamic acid. This protein is found on the exterior of the *E. histolytica* parasite in the form of a trimeric protein complex. KERP1 is a significant factor associated with the virulence of *E. histolytica* and possesses unique characteristics that differentiate it from other known proteins. This unique protein comprises 25% lysine and 19% glutamic acid residues. Its initial discovery was prompted by its interaction with the brush border of human enterocytes (Perdomo et al. 2013). KERP1 localizes to the trophozoite plasma membrane and is closely associated with intracellular vesicles. Gene expression studies have revealed higher levels of KERP1 transcripts in virulent strains and lower protein levels in nonvirulent *E. histolytica* strains. In vivo, research using the hamster model of amoebic liver infection supported the role of KERP1 as a virulence factor Perdomo et al. 2013, Seigneur et al. 2005, Santi-Rocca et al. 2008). Using antisense methods to decrease KERP1 expression halted liver abscess formation, highlighting significance of the protein in amoebic pathogenicity. While the precise function of KERP1 during infection remains unclear, it is undoubtedly engaged in trophozoite interactions, promoting host cell death, phagocytosis, and initiating inflammation in ALA development Nozaki and Bhattacharya 2015). Therefore, KERP1 is considered a crucial virulence factor for *Entamoeba*. The kerp1 gene from *E. moshkovskii* displays homology with the corresponding gene in *E. histolytica*. A study conducted by Weedall in 2020 has unveiled that the kerp1 genes of *E. histolytica* (EHI_098210), *E. nuttalli* (ENU1_189420), and *E. moshkovskii* (EMO_099600) exhibit noteworthy similarities. Specifically, there is a 100% self-match in *E. histolytica*, a 97% amino acid identity across the entire protein in *E. nuttalli*, and a 45% amino acid identity over a portion of the protein in *E. moshkovskii*. In vivo investigations using the hamster model of amoebic liver infection have provided additional evidence supporting the significance of KERP1 as a virulence determinant. Employing an antisense tEmchnique to reduce kerpl expression effectively hindered the development of liver abscesses, underscoring the critical role of this protein in the pathogenicity of amoebae. While the precise role of KERP1 during infection remains elusive, it is clear that this protein plays a role in the interactions between trophozoites and host cells. These interactions are related to adherence, which ultimately results in the death and phagocytosis of host cells. Furthermore, KERP1 appears to be implicated in initiating inflammation during the development of ALA (Nozaki and Bhattacharya 2015)

Amoebapore C (apc) is another gene implicated in the virulence of *E. histolytica*. Earlier research has unveiled the presence of Single Nucleotide Polymorphisms (SNPs) situated in the upstream region of the Amoebapore C gene within *E. histolytica*. These SNPs exhibit a notable connection with the disease outcomes of Amoebiasis. Despite the fact that the precise role of Amoebapore C remains somewhat subtle, its influence on the severity of the disease is becoming clearer through these genetic associations. This gene exhibits homologous counterparts in various other *Entamoeba* species, including *E. dispar* strain SAW760 (EDI_206610), E. invadens strain IP1 (EIN_133650), and *E. moshkovskii* Laredo (EMO_119370). Given its association with disease severity, Amoebapore C presents itself as a promising candidate for inclusion in our genotyping investigation.

The third gene analyzed in the MLST study is the Chitinase of *E. moshkovskii*. Within *Entamoeba* species, there are multiple Chitinases that share a conserved type 18 glycohydrolase domain (Vega et al. in 1997). The process of amoebic encystation involves the expression of Chitinase (Vega et al. in 1997). These Chitinase genes contain repetitive DNA sequences that display notable variations among isolates. Specifically, the repeat types and arrangement patterns within *E. histolytica* show considerable inter-isolate diversity. While the involvement of Chitinase (EC 3.2.1.14) in cyst wall formation is plausible, its role remains unverified. Chitinase functions by breaking down Chitinase, a polymer made up of N-acetyl D-glucosamine units joined by β-1,4 linkages. Although there is a suggestion that *Entamoeba* Chitinase contributes to cyst wall modification during encystation, supporting evidence is limited (Mi-Ichi et al., 2021). In our MLST study, we have included a potential genetic marker: a Chitinase gene from *E. moshokskii* (EMO_056190), which shares sequence similarity with *E. histolytica* (KM1_098160).

The objective of the MLST analysis was to identify genotypes that exhibit a statistical correlation with the co-infection status of *E. moshkovskii*, drawing insights from our epidemiological dataset. We conducted comparative genetic assessments of distinct *E. moshkovskii* populations within diverse co-infection subgroups. We also intend to uncover genetic markers, such as single nucleotide polymorphisms (SNPs), that display significant connections with the occurrence of diarrhoeal episodes attributed to *E. moshkovskii* infections. It is essential to investigate the correlation between *E. moshkovskii* genotypes and infection status in order to acquire a better understanding of the molecular mEmchanisms that play a role in *E. moshkovskii* pathogenesis. Furthermore, it is important to gather genome information of

infecting strains from endemic areas worldwide to expand our understanding of this relationship.

In this chapter, we studied SNPs and the genetic diversity of *E. moshkovskii* isolates from various coinfected individuals. This research will investigate how parasite genotypes relate to different coinfected groups.

6.2. Methodology

6.2.1. Samples

The study utilizes the 68 samples that tested positive for *E. moshkovskii*. These samples were previously characterized through the 18S rRNA locus in the preceding chapter. They were employed in this study for the purpose of conducting multi-locus sequence typing analysis.

6.2.2. PCR amplification:

A total of 68 samples positive for *E. moshkovskii* were chosen for the purpose of amplifying three target genes: KERP1, amoebapore C (apc), and Chitinase. The amplification process was conducted using a reaction mixture with a volume of 50 µl. This mixture included 5 units of TaKaRa Ex-Taq polymerase, PCR buffer at a 1X concentration, 0.2 µM of both forward and reverse primers, and 3 µl of stool DNA samples with a concentration of 50 ng/µl. The amplification reactions were performed using a thermal cycler PCR system from Applied Biosystems. The PCR cycling procedure commenced with an initial denaturation phase at 94°C, lasting for 5 minutes. This was followed by 35 amplification cycles, each comprised of distinct steps. These cycles consisted of a denaturation step at 94°C for a duration of 30 seconds, an annealing phase at 56°C (for KERP1) for 25 seconds, a polymerization step at 72°C lasting 45 seconds, and a concluding extension stage at 72°C, maintained for 7 minutes. The amplification process for apc and Chitinase followed a similar pattern, with the exception of the annealing temperature set at 57°C and the polymerization time reduced to 35 seconds.

Primer sequences, expected PCR product sizes and annealing temperatures employed are provided in **Table 6.1**. The amplified PCR products were separated by agarose gel (Seakem® LE Agarose, Lonza) electrophoresis and observed under UV-transilluminator after 0.5µm/ml ethidium bromide staining.

Table 6.1 Primer sequences, expected PCR product sizes and annealing temperatures of the targeted loci.

Name of the primer	Primer sequence (5’-3’)	Annealing temperature	Product size
Emkerp1_F	TATGAGCGTTGGGGAGATTC	56°C	594 bp
Emkerp2_R	CTTCCCGCCATCAAAAATAA		
Emapc_F	TCTTGAAAGTCTTTGCGCCA	57°C	449 bp
Emapc_R	TCCTCCTCTCGTAGTCCAAA		
EmChitinase_F	TGTGGTGTTTCAAAAGTTTCCA	57°C	357 bp
EmChitinase_R	CAACACAAAAATAAATAGTCATTCACG		

6.2.3. DNA sequencing

PCR products of the expected sizes were extracted using a Roche Gel Extraction Kit following the manufacturer's protocols. Their yield was subsequently verified through gel electrophoresis. The purified PCR products were directly sequenced using the respective amplification primers in both directions, employing the BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, USA. The resulting sequences were analyzed using an ABI3730 sequencer.

6.2.4. Sequence analysis

The obtained DNA sequences were aligned employing the clustalW multiple sequence alignment program of GenomeNet Bioinformatics resources and edited manually. Then the DNA sequences found from the three loci were combined to assign a genotype. The obtained genotypes were also assigned alphanumerical codes. We also identify the SNPs present the obtained local isolates. All the sequences were aligned with reference sequences obtained from AmoebaDB using MultAlin on line tools and thereafter the SNPs were identified. The nucleotide sequences reported in this study have been deposited to NCBI GenBank.

6.2.5. Statistical analysis

GraphPad Prism 9, CA, USA was used for analysing the categorical data. The relationship between the genotypes/repeat patterns and clinical phenotypes was measured using Fisher's exact test. A p-value of < 0.05 was considered statistically significant in all cases.

6.3. Results

6.3.1 Successful amplification of the target loci

Out of the total 68 samples, successful amplification was achieved in 33 samples across three designated target loci (**Fig. 6.1**). Nevertheless, the remaining samples did not yield successful amplification for all three of these loci due to the presence of low DNA concentration in the stool samples. Some samples exhibited faint amplification, which was inadequate for sequencing, while others displayed no distinct bands upon agarose gel electrophoresis. The lack of amplification observed in certain samples could be attributed to either a low cyst concentration of *E. moshkovskii* in faecal samples or issues with the quality of DNA. Furthermore, the existence of genetic polymorphisms at the specified target sites could also contribute to the observed lack of amplification. Although implementing a nested PCR technique has the potential to enhance the amplification rate, we did not prefer to adopt this approach to prevent the risk of cross-contamination. All the obtained sequences have been submitted to NCBI GenBank.

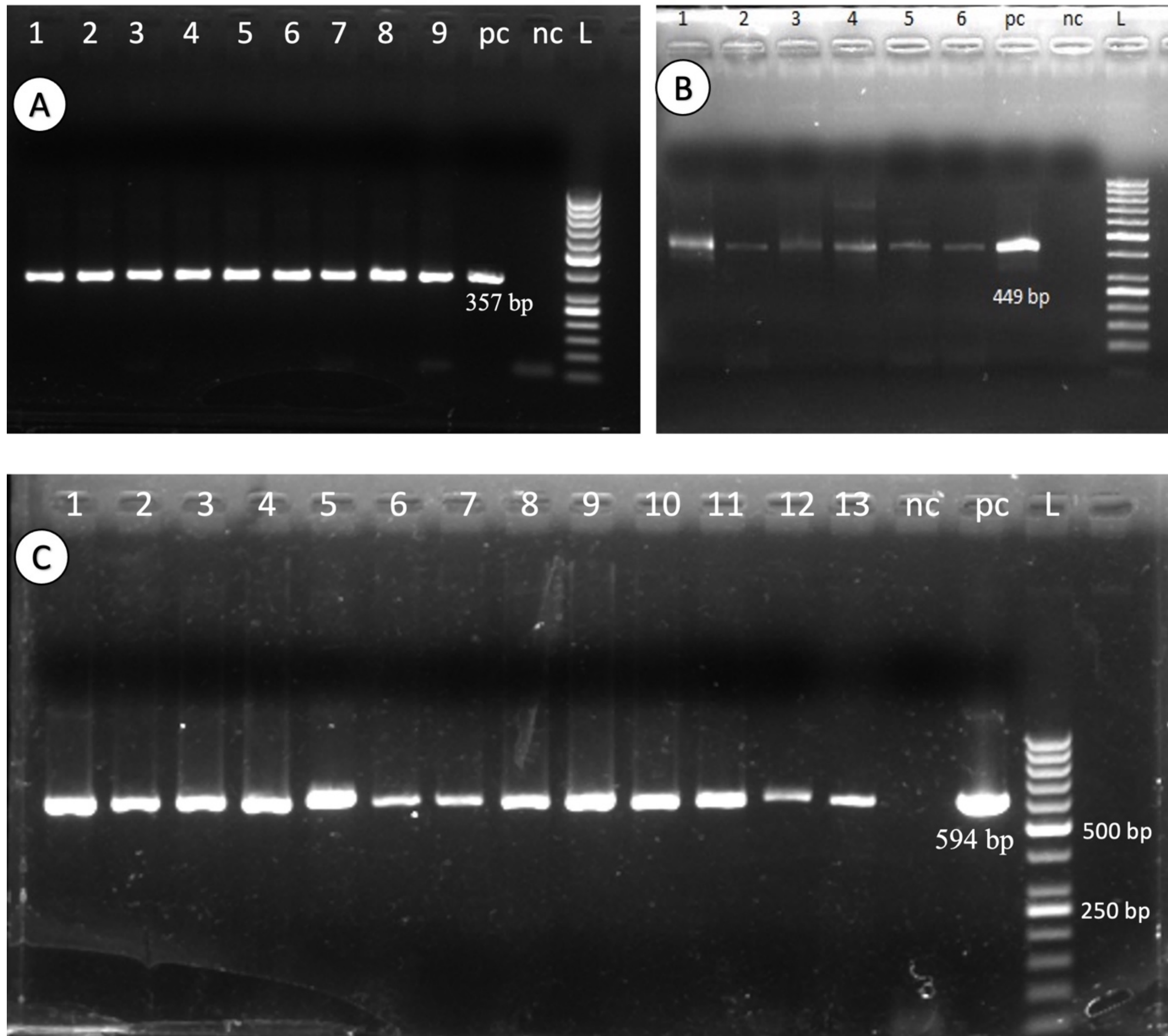


Fig. 6.1 Amplification of different target loci of *E. moshkovskii.* **A.** Chitinase **B.** Amoebapore C **C.** Kerp1. **Nc:** Negative control, **Pc:** Positive control**, L:** 50bp ladder (Fermentas).

6.3.2. Single nucleotide polymorphisms (SNPs)

A total of twenty-three SNPs and three deletions were detected within three genes. We successfully obtained complete gene sequences for the kerp1 and Chitinase genes. In contrast, our analysis of the apc gene involved a partial sequence, encompassing both a partial coding region and a 322 bp-long intron region, as predicted in AmoebaDB.

In Kerp1, we have identified a total of 9 Single Nucleotide Polymorphisms (SNPs) along with three specific deletions denoted as 33A, 42A, and 52A. These deletions resulted in the removal of a sequence of three base pairs, consequently altering the corresponding amino acid sequence. Specifically, the sequence **-Val-Val-Gln-His-Arg-Ala-** was substituted with **-Tryp-Phe-Thr-Gln-Ser-**. As a consequence of the deletion, one amino acid was lost in the replaced sequence. Interestingly, despite this alteration in the amino acid sequence, our analysis did not reveal any statistically significant associations between this deleted amino acid stretch and various coinfected groups. Furthermore, we have identified five SNPs labeled as 23A/C, 25 A/C, 26C/T, 27T/G, and 28G/A. These variants have demonstrated a significant correlation with exclusive incidences of diarrhoea, with corresponding p-values of 0.0261 for each SNP. Conversely, the remaining three SNPs did not exhibit any statistically significant connections with the coinfected groups.

In the apc gene, a total of 10 SNPs were identified. Among these, 9 SNPs were located within the intronic region of the gene. Four intronic SNPs exhibited a statistically significant correlation with the occurrence of sole diarrhoea, which we refer to as the Sole D group (as shown in Table 6.2). Within these four SNPs, three - namely 420T/A (p=0.0224), 564T/A (p=0.2916), and 523A/T (p=0.0538) - demonstrated a positive statistical correlation with the incidence of sole diarrhoea. Additionally, one SNP, 299A/T, displayed a negative association (p=0.0315) with sole diarrhoeal incidence. However, it should be remembered that the intronic SNPs do not have a direct impact on the functionality of the translated protein. As a result, the identified SNPs can only serve as genetic markers. It is likely that they do not play a role in regulating any function within the *E. moshkovskii* cell. It is important to note that the intron region reported in this study was based on the predicted genomic sequence of the apc gene (EMO_119370) sourced from AmoebaDB.

The Chitinase locus analysis revealed the presence of three SNPs, all of which lacked statistical significance when correlated with specific subgroups of *E. moshkovskii*. Details of these identified SNPs within the target loci of the studied isolates can be found in **Table 6.2**.

In the obtained DNA sequences of the three loci, a majority of the SNPs were characterized as non-synonymous mutations.

Table 6.2 Identified SNPs within the target loci of the studied isolates.

Target loci	Base pair (bp) analysed	*SNP position including deletion	Amino acid substitution	Coinfection status			
				Sole D	IEH	IOEP	IB/V
Kerp1	546	Deletion 33A, 42A, 52A	12-17 VVQHRA/ WFT|QS	X^C	X^C	X^C	X^C
		23A/C	8Q/P	p^b=0.0261 X^2=4.90	X^C	X^C	X^C
		25 A/C	9T/L	p^b=0.0261 X^2=4.90	X^C	X^C	X^C
		26C/T	9 T/L	p^b=0.0261 X^2=4.90	X^C	X^C	X^C
		27T/G	11 T/L	p^b=0.0261 X^2=4.90	X^C	X^C	X^C
		28G/A	10V/I	p^b=0.0261 X^2=4.90	X^C	X^C	X^C
		374 A/G	125 E/G	X^C	X^C	X^C	X^C
		405 G/T	135N/K	X^C	X^C	X^C	X^C
		432C/G	144D/E	X^C	X^C	X^C	X^C
		441T/A	147D/E	X^C	X^C	X^C	X^C
Amoebapore C (apc)	407	299A/T	Intron region	p^b=0.0315 X^2= 4.626 (negative association)	X^C	X^C	X^C
		325A/T	Intron region	X^C	X^C	X^C	X^C
		327C/T	Intron region	X^C	X^C	X^C	X^C
		360T/A	Intron region	X^C	X^C	X^C	X^C
		370T/A	Intron region	X^C	X^C	X^C	X^C
		398A/C	Intron region	X^C	X^C	X^C	X^C
		420T/A	Intron region	p^b=0.0224 X^2= 5.215	X^C	X^C	X^C
		564T/A	Intron region	p^b= 0.2916 X^2= 1.112	X^C	X^C	X^C
		523A/T	Intron region	p^b= 0.0538 X^2= 3.718	X^C	X^C	X^C
		607A/T	55K/N	X^C	X^C	X^C	X^C
Chitinase	306	75T/C,	25Y/Y(Synonymous)	X^C	X^C	X^C	X^C
		223A/T	75I/L	X^C	X^C	X^C	X^C
		286T/C	96S/P	X^C	p^b= 0.0001 X^2= 17.22 (negative association)	X^C	X^C

[b] Probability value of the particular association

[c] Does not have any association with disease outcomes,

[d] Synonymous SNPs, do not affect the protein sequences,

[e] intron region.

X^2=Chi-square value

***The positions of all SNPs were reported with reference to first base of the start codon, which is designated as position 1.**

6.3.3. Association between genotype and co-infection status:

After conducting sequencing on all the samples, we identified a total of six distinct haplotypes in the Kerp1 group. Notably, one of these haplotypes, Emk3, displayed a complete 100% match with the reference sequence EMO_099600. In contrast, the apc group also exhibited six distinct haplotypes, all of which differed from the reference sequence EMO_119370. Within the Chitinase gene sequences, we observed three distinct haplotypes. Notably, one of these haplotypes, Emch1, exhibited similarity to the reference sequence EMO_056190. These individual haplotypes were subsequently pooled together to construct the respective genotypes.

After combining four distinct loci, this study has successfully identified 17 distinct genotypes labeled as M1 through M17. Among these genotypes, seven, specifically M1, M3, M5, M8, M9, and M17, were identified in multiple isolates (**Table 6.3**). Notably, genotype M1 exhibited a statistically significant association with the sole diarrhoeal group within *E. moshkovskii* infection, as indicated by a p-value of p=0.0394. While M9 and M17 were observed in the individual coinfected groups Sole D and IOEP, respectively, their presence did not demonstrate any statistically significant associations with their respective groups. The M12 genotype was detected in several groups, except for the Sole D group. The remaining genotypes were found in both the Sole D group and other co-infected groups. This research demonstrated that the M1 genotype holds the highest potential for being a pathogenic strain of *E. moshkovskii.*

Table 6.3 List of identified genotypes (M1 to M17) resulting from the combination of three independent studied loci

Co-infection status	Sample ID	Sequence pattern			Genotype
		Kerp1	Amoebapore C (apc)	Chitinase	
Sole D	EM_IND/1	Emk2	Emapc3	Emch1	[b]M1
	EM_IND/3	Emk1	Emapc5	Emch1	M2
	EM_IND/4	Emk2	Emapc3	Emch1	[b]M1
	EM_IND/5	Emk2	Emapc3	Emch1	[b]M1
	EM_ID/6	Emk1	Emapc6	Emch2	M3
	EM_IND/10	Emk1	Emapc4	Emch1	M4
	EM_IND/11	Emk3	Emapc4	Emch1	[a]M5
	EM_IND/12	Emk2	Emapc3	Emch3	M6
	EM_IND/15	Emk1	Emapc5	Emch2	M7
	EM_IND/16	Emk2	Emapc3	Emch1	[b]M1
	EM_IND/17	Emk1	Emapc1	Emch3	M8
	EM_IND/19	Emk3	Emapc4	Emch2	[a]M5
	EM_IND/21	Emk2	Emapc5	Emch1	[a]M9
	EM_IND/22	Emk2	Emapc3	Emch1	[b]M1
	EM_IND/26	Emk2	Emapc5	Emch1	[a]M9
IB/V	EM_IND/34	Emk1	Emapc6	Emch2	[a]M3
	EM_IND/36	Emk2	Emapc3	Emch1	[b]M1
	EM_IND/37	Emk3	Emapc4	Emch2	[a]M5
	EM_IND/39	Emk1	Emapc3	Emch1	M10
	EM_IND/40	Emk4	Emapc6	Emch1	M11
	EM_IND/47	Emk1	Emapc1	Emch1	[b]M12
	EM_IND/48	Emk3	Emapc1	Emch1	M13
	EM_IND/49	Emk1	Emapc1	Emch1	[b]M12
	EM_IND/50	Emk5	Emapc6	Emch1	M14
	EM_IND/51	Emk2	Emapc6	Emch2	M15
IEH	EM_IND/57	Emk1	Emapc1	Emch3	[a]M8
	EM_IND/60	Emk2	Emapc1	Emch3	M16
	EM_IND/61	Emk3	Emapc4	Emch1	[a]M5
IOEP	EM_IND/63	Emk3	Emapc2	Emch2	[a]M17
	EM_IND/64	Emk1	Emapc1	Emch3	[b]M8
	EM_IND/65	Emk1	Emapc1	Emch1	[b]M12
	EM_IND/67	Emk6	Emapc2	Emch2	[a]M17
	EM_IND/68	Emk1	Emapc6	Emch2	[a]M3

[a]multiple occurrences but not statistically significant.
[b]Statistically associated with the Sole D group, $p=0.0394$ ($X^2=4.244$).

6.3.4. Genetic diversity:

The range of haplotype diversity (Hd) among individual polymorphic loci ranges from 0.572 to 0.833. The number of haplotypes varies between 2 and 6 within the 33 *E. moshkovskii* samples. Among the three examined loci, the apc locus showed with the highest Hd value of 0.833 and the highest number of haplotypes (6). Additionally, the apc locus exhibits the highest observed nucleotide diversity. Moderate levels of haplotype diversity were revealed within the kerp1 and Chitinase loci, with Hd values of 0.572 and 0.589, respectively (**Table 6.4**). Additionally, these loci exhibited moderate levels of nucleotide diversity (π), with kerp1 observing 0.0048 and Chitinase observing 0.0035. Among the loci examined, the apc locus showed the greatest number of polymorphic sites (10), while the Chitinase locus showed the fewest (3). Tajima's D statistics revealed positive values for all of the loci. These positive values could imply the presence of either a population bottleneck or balancing selection. The presence of a substantial variety of genotypes within this group, coupled with the positive Tajima's D value, may support the hypothesis of balancing selection (**Table 6.4**). However, given that these values did not show statistical significance, confirming these results would require a larger sample size.

The combined nucleotide sequences of the three target loci were either 1259 bp (Due to 3 deletions in Kerp1) or 1256 bp long and included 22 variable sites. The Hd value was 0.93. Tajima's D statistic for the concatenated sequences was 1.42, supporting the idea of balancing selection (**Table 6.5**). To validate these findings further, a larger sample size would be needed.

6.3.5. Linkage disequilibrium (LD) and recombination analyses of target loci

We assessed intragenic LD and the count of potential recombination events for each target locus. At the apc locus, an incomplete intragenic LD value was observed ($|D'|$ Y = 0.9779 - 0.1659X), with Y representing the LD value and X indicating the nucleotide distance in kilobases. The incomplete intragenic LD value at the apc locus suggests a non-random distribution of its alleles within the *E. moshkovskii* population. Conversely, complete LD values ($|D'|$ Y = 1.0000 – 0.0000X) were discovered at the Kerp1 and Chitinase loci, indicating a random distribution of alleles for these two genes. The analysis of intragenic recombination revealed a single potential event (Rm) exclusively at the apc locus. Conversely, no recombination events were observed at the Kerp1 and Chitinase loci, signifying that the alleles of these two genes are distributed randomly in the studied population (**Table 6.4)**.

Our study isolates were analyzed for an overall interlocus LD and the number of potential recombination events. The concatenated multilocus sequence data was used for this purpose. The analysis revealed an incomplete LD value (|D′| Y=0.8169 + 0.1059X) with a single potential recombination event in the population. This was observed when the concatenated sequences of all three loci were analyzed. An incomplete interlocus LD value (|D′| Y = 0.9928–0.0027×) was discovered between Chitinase and Kerp1, indicating a single recombination event. However, upon further analysis of concatenated sequences among apc + Chitinase and apc + Kerp1, two potential recombination events with incomplete interlocus LD values were observed (**Table 6.6**). This interesting finding suggests a possible non-random association of the apc locus with both Chitinase and Kerp1.

Table 6.4 Different genetic diversity indices of *E. moshkovskii* population based on three target loci.

	Haplotype details		**Nucleotide details**		**Number of segregating sites (S)**	**Tajima's D**	***LD (|D′|)***	**Rm**
	Number of haplotype	*Hd*	*K*	*π*				
Kerp1	5	0.572	2.59	0.0048	9	0.51	Y=1.0000 + 0.000X	0
apc	6	0.833	3.97	0.0097	10	1.90	Y=0.9779 - 0.1659X	1
Chitinase	3	0.589	1.08	0.0035	3	1.06	Y=1.0000 + 0.000X	0

Assessment of intragenic recombination revealed that apc exhibited one recombination event (Rms), and LD was incomplete in this loci, whereas the remaining two markers (with complete LD) did not have Rms.

Table 6.5 Different genetic diversity indices of *E. moshkovskii* population based on using concatenated multilocus sequences.

	Haplotype details		**Nucleotide details**		**Number of segregating sites (S)**	**Tajima's D**	***LD (|D′|)***	**Rm**
	Number of haplotype	*Hd*	*K*	*π*				
Kerp1+ apc+ Chitinase	17	0.93	7.65	0.0060	22	1.42	Y=0.8169 + 0.1059X	1

6.3.6. Interlocus LD and recombination analyses of *E. moshkovskii* population from different co-infection/Sole infection groups

We conducted Interlocus LD and recombination analyses on the *E. moshkovskii* population from four groups (Sole D, IBV, IEH, and IOEP) using concatenated multilocus sequence data. Our analysis revealed that only the apc locus showed a single recombination event in intralocus LD analysis. To further analyze interlocus LD values, we looked at concatenated multilocus sequences both including and excluding the apc locus. Interestingly, we found that the inclusion of the apc locus increased recombination events. However, LD analysis of concatenated multilocus sequences from the IEH and IOEP groups produced a complete interlocus LD value (| D′| Y = 1.0000 + 0.0000×) with no recombination events in either case. Based on the complete interlocus LD value (|D′| Y = 1.0000 + 0.0000×) and absence of recombination events in the IEH and IOEP groups, it appears that these groups might be isolated compared to the others. However, since most concatenated sequences show at least one recombination event, it is possible that inter-population genetic recombination occurs among the different subpopulations. One notable findings from this study is that the IEH and IOEP population of *E. moshkovskii* are undergoing a speciation process due to their isolation (**Table 6.7 and Table 6.8**).

Table 6.6 Different genetic diversity indices, interlocus linkage disequilibrium (LD) and recombination analyses of *E. moshkovskii* population using concatenated multilocus sequences.

		Haplotype details		**Nucleotide details**		**Number of segregating sites (S)**	**Tajima's D**	***LD* (|*D′*|)**	**Rm**
		Number of haplotype	*Hd*	*K*	*π*				
Including apc	**apc+ Chitinase**	11	0.90	3.97	0.0071	13	1.87	Y=0.9708 - 0.4183X	2
	apc+ Kerp1	13	0.89	6.56	0.0069	19	1.37	Y=1.067 - 0.5529X	2
Excluding apc	**Chitinase+ Kerp1**	9	0.83	3.67	0.0043	12	0.776	Y=0.7682 + 0.4469X	1

Table 6.7 Interlocus LD and recombination investigations within *E. moshkovskii* population across various co-infection/sole infection groups, utilizing combined multilocus sequences excluding apc loci.

	Populations	No. of samples	No. of polymorphic sites analyzed	No. of pairwise comparisons	No. of significant pairwise comparisons (Fisher's exact test)	*LD* ($\|D'\|$)	Rm
Excluding apc	**All**	33	12	66	12	Y=0.7682 - 0.4469X	1
	Sole D + IBV	25	10	45	11	Y=0.7121 - 0.5057X	1
	Sole D + IEH	18	8	28	11	Y=0.7423 - 0.8854X	1
	Sole D + IOEP	20	10	45	21	Y=0.8196 - 0.4000X	1
	IBV + IEH	13	10	45	11	Y=0.6397 - 0.4699X	1
	IBV + IOEP	15	12	66	11	Y=0.8170 - 0.2243X	1
	IEH + IOEP	8	10	45	1	Y=1.0000 - 0.0000X	0

Table 6.7 Interlocus LD and recombination investigations within *E. moshkovskii* population across various co-infection/sole infection groups, utilizing combined multilocus sequences after inclusion of apc loci.

	Populations	No. of samples	No. of polymorphic sites analyzed	No. of pairwise comparisons	No. of significant pairwise comparisons (Fisher's exact test)	*LD* ($\|D'\|$)	Rm
Including apc	**All**	33	22	231	51	Y=0.8169 + 0.0398X	3
	Sole D + IBV	25	20	190	40	Y=0.8280 + 0.0717X	3
	Sole D + IEH	18	18	153	46	Y=0.9681 - 0.6813X	3
	Sole D + IOEP	20	20	190	60	Y=0.8645 + 0.0426X	3
	IBV + IEH	13	20	190	28	Y=0.8253 - 0.1884X	2
	IBV + IOEP	15	22	231	38	Y=0.8806 + 0.1126X	2
	IEH + IOEP	8	19	171	16	Y=1.0000 - 0.0000X	0

A clear observation from the analyses of both intragenic and interlocus LD was that genetic recombination predominantly took place at the apc locus.

6.4. Discussion

Our goal was to analyze the genetic makeup of various isolates of *Entamoeba moshkovskii* and its association with virulence factors found in *Entamoeba histolytica*. We focused specifically on the loci of lysine and glutamic acid-rich protein 1 (KERP1), amoebapore C (pore-forming peptides), and Chitinase. KERP1 is found on the trophozoite plasma membrane and internal vesicles, where it plays a crucial role in establishing amoeba-cell contacts and the development of liver abscesses. Amoebapore forms ion channels or pores in lipid membranes, depolarizing target cells. Chitinase, on the other hand, breaks down chitinase, a β-1,4-linked polymer of N-acetyl-d-glucosamine, and is believed to be involved in remodelling the cyst wall during encystation in *Entamoeba*. In our study, we employed PCR amplification using specific primers designed for targeting these genetic loci. This approach has the potential to provide novel insights into the co-infection dynamics of *E. moshkovskii*.

Accurate identification and genetic characterization of clinical isolates from endemic regions worldwide provides a valuable tool for understanding the impact of parasite genome on the outcomes of amoebic infections. Previous research has established that tRNA-linked STR loci serve as surrogate markers for determining disease outcomes (Ali et al. 2012). In our current research, we have genetically characterized *E. moshkovskii* populations with varying co-infected groups using the abovementioned coding genes. The Kerp1 gene exhibited the highest number of SNPs, with four of them being associated with diarrhoea incidence and potentially serving as genetic markers. These SNPs may also play a role in modulating the pathogenicity of *E. moshkovskii*. The Apc gene displayed a number of significant SNPs, the exact impact of which remains to be determined. However, the presence of these SNPs can serve as a genetic marker for diarrhoeal diseases caused by isolates similar to those observed in Kerp1. Most of the SNPs observed in our study were non-synonymous, which aligns with previous findings for Mycobacterium tuberculosis, where only 36 out of 101 identified SNPs were synonymous (Baker et al. 2004). This trend was also noted by Das et al. 2021 in their study of Multilocus sequence typing (MLST) of *Entamoeba histolytica*. Interestingly, the Chitinase gene showed only synonymous SNPs and was found to be the most conserved gene among the studied loci, with only three SNPs observed. In contrast, the Chitinase of *E. histolytica* is highly polymorphic and contains STR units, whereas the Chitinase of *E. moshkovskii* is not a highly polymorphic gene as observed in this study (Das et al. 2014). The reported SNPs could potentially play a role in drug sensitivity in *E. moshkovskii*, similar to how certain SNPs have

been linked to multidrug resistance in *Plasmodium falciparum*, as reported by Coulibaly et al. in 2022 (Coulibaly et al. 2022).

Recombination events were only identified in the apc locus. The presence of co-infection specific SNPs, potential recombination events within the apc locus, and various non-synonymous base changes all suggest that this region of the genome is under selection pressure. As such, these observations may indicate that apc could play a crucial role in determining the virulence of *E. moshkovskii*. The precise function of apc in *E. moshkovskii* pathogenesis remains unknown. While investigating another gene, Kerp1, we identified a notable quantity of SNPs, despite the absence of detected potential recombination events. Therefore, further studies with a larger sample size are required to gain a better understanding of role of Kerp1 in population structure of *E. moshkovskii*. It is important to note that no studies to date have been conducted on the role of different genes in *E. moshkovskii* pathogenicity.

The findings of the study suggest a correlation between the parasite genotypes and *E. moshkovskii* infection status. This study is the first to explore the direct link between parasite factors and the infection dynamics of *E. moshkovskii*. However, further biomarkers are necessary to comprehensively understand the role of parasite genome.

6.4. Conclusion

The latest research has unveiled the genetic composition of *E. moshkovskii* isolates under investigation, establishing their link to infection dynamics. Through the analysis, numerous significant SNPs within specific genetic regions have been detected. These SNPs exhibit the potential to influence, either directly or indirectly, the pathogenicity and drug sensitivity of *E. moshkovskii*. The investigation has also pinpointed distinct clusters of isolates that display genetic segregation. Moreover, the study supports the hypothesis that a connection exists between parasite genotypes and infection dynamics.

CHAPTER VII

OCCURRENCE OF *ENTAMOEBA MOSHKOVSKII* IN PIGS WITH ZOONOTIC POTENTIAL FROM EASTERN INDIA

Occurrence of *Entamoeba moshkovskii* in pigs with zoonotic potential from eastern India

7.1. Background

In the previous chapters, we observed that *Entamoeba moshkovskii* is an enteric pathogen with the potential to cause diarrhoeal incidents in the study area. We have identified certain genotypes that are significantly associated with diarrhoea when present as the sole infection. While the prevalence of *E. moshkovskii* in diarrheal patients is notably high, the exact transmission route and environmental reservoir of the species remain unknown. *E. moshkovskii* is an amphizoic organism, capable of surviving in the environment and also surviving as a commensal in the human intestine. Understanding the transmission of this parasite is crucial for controlling the parasitic infection. Currently, the animal reservoir that may serve as a potential host for *E. moshkovskii* remains unknown. This chapter aims to undertake a comprehensive study to discover any potential animal reservoirs associated with *E. moshkovskii*.

The genus *Entamoeba* (Casagrandi & Barbagallo, 1897) consists of at least nine species: *E. histolytica*, *E. dispar* (Brumpt, 1925), *E. moshkovskii*, *E. bangladeshi* (Royer, 2012), *E. coli* (Grassi, 1879), *E. nuttalli* (Catellani, 1908), *E. hartmanni* (Prowazek, 1912), *E. polecki* (Von Prowazek, 1912) and *E. chattoni* (Levine, 1961), which are able to reside in the human intestinal lumen (Fotedar et al. 2007, Delialioglu et al. 2008, Ngui et al. 2012). The first three species are the most prevalent and are morphologically similar under light microscope, but have different biochemical and genetic characteristics (Verweij et al. 2003). Although *E. polecki* has rarely been implicated as a cause of diarrheal disease, it is important to keep in mind that most species are usually believed as commensal organisms of the human gut except *E. histolytica* and *E. moshkovskii* (see Salakai et al. 1979, Ali et al. 2007, Al-Areeqi et al. 2017). *Entamoeba histolytica* is considered the most recognized pathogen of the human gut (Ali et al. 2007).

The role of *E. moshkovskii* as an etiological agent of diarrhoea in humans remains unclear (Clark and Diamond 1991). However, our study described in the earlier chapters has reported the association of *E. moshkovskii* with gastrointestinal clinical manifestations indicating *E. moshkovskii* might be associated with pathogenicity (Ali et al. 2007, Fotedar et al 2007b, Khairnar and Parija 2007, Ngui et al. 2012).

Entamoeba moshkovskii in human stool samples has been detected in many countries such as United States, Iran, Turkey, Italy, Australia, Bangladesh, India (Pondicherry), Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil, but no data are available about the occurrence of *E. moshkovskii* in farm animals. Although a recent study reported the presence of *E. moshkovskii* in non-human primates (NHP), data on the prevalence of *E. moshkovskii* in others animals are limited (Levecke et al. 2010). Therefore, the role of these animals as a potential reservoir for zoonotic transmission of *E. moshkovskii* remains unclear. Most of the previous studies were conducted for detection of cysts or trophozoites in human stools via light microscopy or PCR. As a result, the distribution and natural host range of *E. moshkovskii* are largely undetermined.

Pai et al. reported the occurrence of *E. histolytica/E. dispar* on the cuticle and/or digestive tract of American cockroaches (*Periplaneta americana* Linnaeus, 1758) and in the digestive tract of German cockroach (*Blattella germanica* Linnaeus, 1767) in South Taiwan, 2003 (Pai et al. 2003). Some non-human primates cats, and dogs have also been identified as natural hosts of *E. histolytica* (Muriuki et al. 1998). Other study showed that two species, *E. suis* and *E. polecki* have been identified in pigs (Ji et al. 2019). *Entamoeba suis* is mostly restricted to pigs and causes hemorrhagic colitis by breaking down the lamina propria (Matsubayashi et al. 2014). Whereas *E. polecki* causes infection in many natural hosts, including humans, non-human primates and pigs, the epidemiology of its infections and particularly the role of non-human hosts as a potential reservoir for zoonotic transmission remain unclear (Muriuki et al. 1998, Tachibana et al. 2009). In India, swine husbandry is on the rise and it plays a crucial role in livestock farming. The risk of exposure to zoonotic parasitic agents such as *Entamoeba* spp. from pigs is thus predictable. In this study, we report the occurrence of *E. moshkovskii* in stool samples from indigenous pig breeds farmed in and around Kolkata, West Bengal. This study suggests the potentiality of swine as a reservoir for *E. moshkovskii* that might be a route for its zoonotic transmission.

7.2. Methodology

7.2.1. Study area and population

A total of 294 fresh faecal specimens were collected between February 2018 and December 2019 from five different regions in Kolkata, West Bengal, India. All specimens were collected from piglets (< 4 weeks old), weaners (1–3 months old), porkers (3–9 months old)

and hogs (> 12 months old) immediately after defecation on the ground. From each of the animal pen one sample was collected.

7.2.2. Sample collection and microscopy

Faecal consistency, sex and developmental stage of pig were recorded at the spot of sample collection. Faecal samples were transported to the laboratory in ice boxes within 2 hours and preserved in at 4 °C before microscopic analysis. After microscopy, DNA isolation was done generally within 48 hours of sample collection. A portion of each sample was examined microscopically in both saline wet mounts and Lugol's iodine wet mounts for cysts of amoebae.

7.2.3. DNA extraction

Total genomic DNA was isolated directly from approximately 200 mg of each fecal sample that was found positive for cysts of *Entamoeba* spp. through microscopy, using STOOL DNA Minikit (QIAGEN, USA) as per manufacturer's instructions. The isolated DNA was kept at -20 °C until molecular identification of *E. moshkovskii*.

7.2.4. PCR amplification

For detection of the SSU-rDNA gene of *E. moshkovskii* in faecal DNA, a set of previously described nested primers was used: Em_F1 5'CTCTTCACGGGGAGTGCG-3', Em_R1 5'TCGTTAGTTTCATTACCT-3', Em_nF2 5'GAATAAGGATGGTATGAC-3' and Em_nR2 5'AAGTGGAGTTAACCACCT-3'. Primary PCR for *E. moshkovskii* was performed in a final volume of 50 µl mixture 50 µl contained 1X PCR buffer, 4.0 mM MgCl2, 0.2 mM of each dNTP, 0.1 M of each forward and reverse primer (Em_F1 & Em_R1), 1U of Taq polymerase (Bioline), 3 µl of stool DNA samples at 50 ng/µl concentration. The PCR cycle was as follows, for primary PCR: 96 °C for 5 minutes followed by 35 cycles each consisting of 94°C for 45 seconds, 60°C for 40 s and 72°C for 45 s, followed by a final extension at 72°C for 7 min. Successively, 1.5 µl of primary PCR products were subjected as a template for the nested PCR. Amplification was achieved using the as described above for primary PCR except for MgCl2 concentration and annealing temperature. MgCl2 concentration was 1.5 mM and annealing temperature was 55°C for the nested PCR cycle. Nested PCR generated amplicons of 258 bp in the presence of *E. moshkovskii*. Amplified PCR products after nested PCR were separated by agarose gel electrophoresis and visualised in a UV transilluminator after 0.5 µm/ml of ethidium bromide staining.

7.2.5. Restriction fragment length polymorphism (RFLP) analysis

The nested PCR products were confirmed by RFLP. The restriction enzyme MboII recognises and cleaves DNA at 5'…GAAGA(N)8∨…3'/3'…CTTCT(N)7^…5', producing a single 3'-protruding nucleotide in the case of *Entamoeba* spp. (Furmanek-Blaszk et al. 2009). This recognition site is present at 206/205 position in the amplicon of *E. moshkovskii* 18S rDNA locus. Around 120–160 ng of PCR products were digested with Mbo II (NEB) in a total volume of 20 µl reaction mixture at 37°C for 1 hour, followed by heat inactivation at 65°C for 20 min. Digested PCR products were separated by 1.8% agarose gel electrophoresis and visualized in a UV transilluminator after 0.5 µm/ml of ethidium bromide staining.

7.2.6. DNA sequencing

Positive PCR products were sequenced to test the specificity of the performed PCR assay. PCR products were purified using the Roche PCR Gel extraction Kit and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a DNA analyzer (Applied Biosystems, Foster City, CA, USA).The accuracy of the sequence was verified by using both forward and reverse primer separately during sequencing in 5'-3'direction. The obtained sequences were aligned using ClustalW multiple sequence alignment program of GenomeNet Bioinformatics tools and edited manually. The obtained 18S rRNA gene sequences of *E. moshkovskii* compared to those available in the GenBank database using the BLAST program run on the NCBI website.

7.2.7. Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (-389.77) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1258)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). We opted four known sequences of *E. histolytica* (KJ870211.1), *E. dispar* (AB282661.1), *E. bangladeshi* (KR025412.1) and E. nutalli (AB749447.1) to construct the phylogenetic tree of the *E. moshkovskii* isolates obtained in this

study. These four species were chosen because they are the closest species of *E. moshkovskii*. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 nucleotide sequences. There was a total of 222 positions in the final dataset Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

The obtained nucleotide sequences in this study have been submitted to NCBI GenBank database.

7.2.8. Statistical analysis

GraphPad prism v.8.4.2, CA, USA was used to analyse the data. The relationship between the occurrence of *E. moshkovskii* with others variables like faecal consistency, age, sex and developmental stage were measured by testing X2. In all cases differences were considered statistically significant when p-value was less than 0.05.

7.3. Results

7.3.1 Occurrence of *Entamoeba moshkovskii* in pigs

Microscopic examination of 294 faecal samples revealed that 87.8% (n/N = 258/294, 95% CI 83.5–91.0%) were cysts/trophozoites positive for *Entamoeba* spp. (**Fig. 7.1**). All the 258 microscopy-positive samples obtained from microscopy were subjected to PCR for detection of *E. moshkovskii*. A total of 16 out of 258 samples were positive for *E. moshkovskii*, detected by nested PCR assay. Therefore, 6.2% (n/N = 16/258, 95% CI 3.8–9.9%) of all the *Entamoeba* spp. infected samples contained *E. moshkovskii* DNA. Overall, 5.4% (n/N = 16/294, 95% CI 3.4–8.7%) samples were detected as *E. moshkovskii* positive among all the samples collected. **Fig 7.2** displays the gel documentation image capturing the results of the molecular screening process.

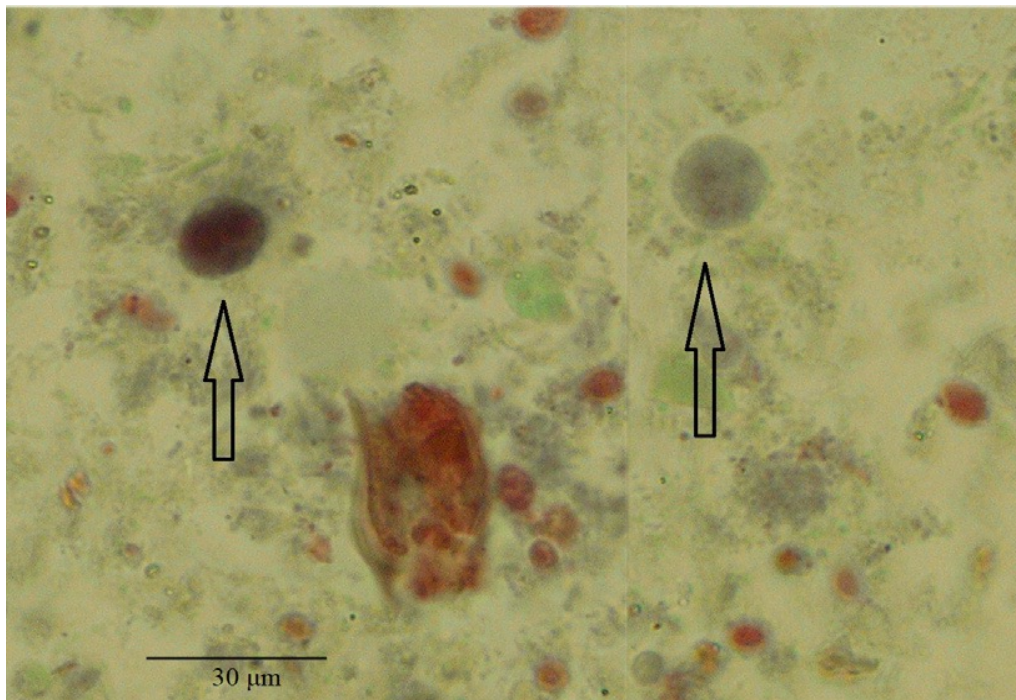


Fig. 7.1 Microscopic view of cysts of *Entamoeba* spp. cysts after iodine staining. (40X).

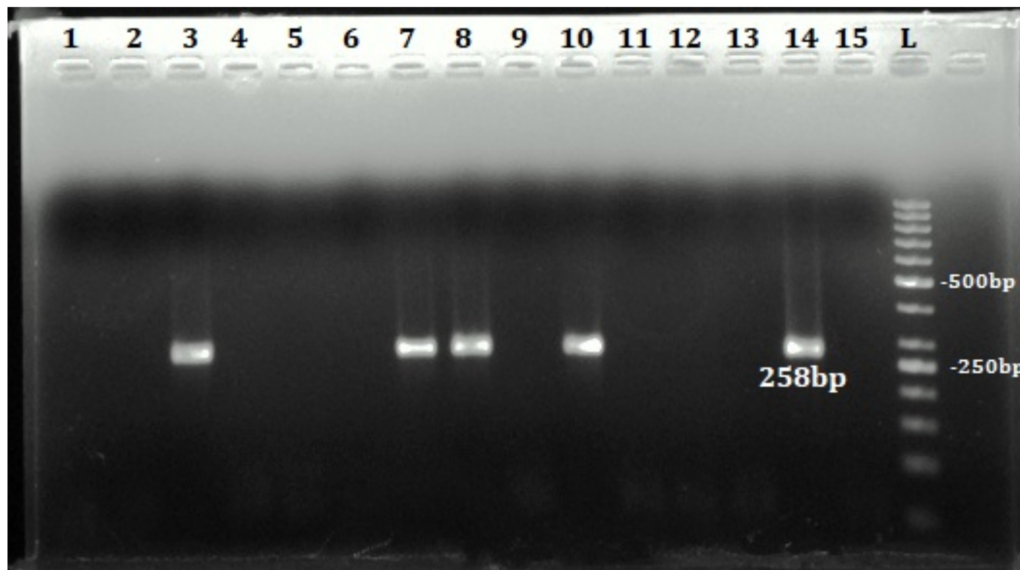


Fig. 7.2 Amplification of the 18S rDNA locus of *Entamoeba moshkovskii* in pig stool samples collected from Kolkata and adjacent areas. The PCR product size is 258bp. Lane 1 to 13 represents pig stool samples, while Lane 14 shows the positive control containing *E. moshkovskii* DNA. Lane 15 serves as the negative control. Positive amplification for *E. moshkovskii* is observed in Lanes 3, 7, 8, and 10.

7.3.2. Distribution of *E. moshkovskii* infection in pigs

The occurrence of *E. moshkovskii* infection in pigs was higher in males (n/N =10/163, 6.1%, 95% CI 3.4–10.9%) than females (n/N = 06/131, 4.6%, 95% CI 2.1–9.6%) but the difference was not statistically significant (X2=0.341, df-1, p=0.56) (**Fig. 7.3**). The association of *E. moshkovskii* infection in swine was not statistically significant (X2=0.013, df-2, p=0.994) in terms of fecal consistency (**Fig. 7.4**). It was also observed that the *E. moshkovskii* infection in pigs were not significantly associated (X2=3.43, df-3, p= 0.33) with their developmental stage (**Fig. 7.5**). However, the highest (n/N=7/73, 9.6%, 95% CI 4.7%-18.5%) and lowest (n/N=1/38, 2.6%, 95% CI 0.1%-13.5%) infection of *E. moshkovskii* was found among porkers and piglets respectively. However, a specific seasonal pattern was not observed for *E. moshkovskii* infection in pigs (data not shown). The detailed data of distribution of *E. moshkovskii* with their statistical association are shown in **Table 7.1**. This result demonstrates that swine can host *E. moshkovskii* and might act as a potential natural reservoir for *E. moshkovskii*.

Table 7.1 Distribution of *Entamoeba moshkovskii* Tshalaia, 1941 in pigs according to their developmental stage, sex and faecal consistency.

Variables		No. examined	No. positive	Prevalence	95% CI	X^2	p value	df
Developmental stage	Piglets (<4 weeks old)	38	1	2.94%	00.14%-13.50%	3.43	0.33	3
	Weaners (1–3 months old),	68	3	4.41%	01.20%-12.19%			
	Porkers (3–9 months old)	73	7	9.59%	04.72%-18.50%			
	Hogs (>12 months old)/Adult	115	5	4.34%	01.87%-09.78%			
Sex	Male	163	10	6.13%	03.36%-10.92%	0.341	0.56	1
	female	131	6	4.60%	02.11%-09.63%			
Fecal consistency	Firm feces	143	8	5.59%	02.86%-10.65%	0.013	0.994	2
	Soft feces	94	5	5.31%	02.29%-11.85%			
	Liquid feces	57	3	5.26%	01.44%-14.37%			

X^2- Chi-square value; df- Degrees of freedom; CI- Confidence Interval

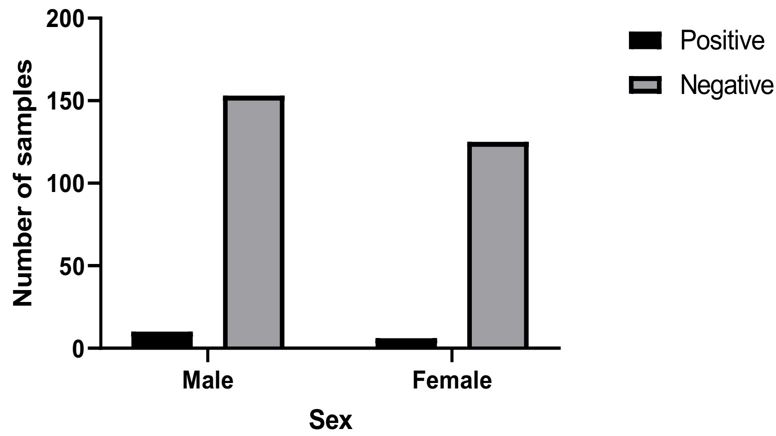


Fig. 7.3 Occurrence of *E. moshkovskii* infection in Male and female pigs

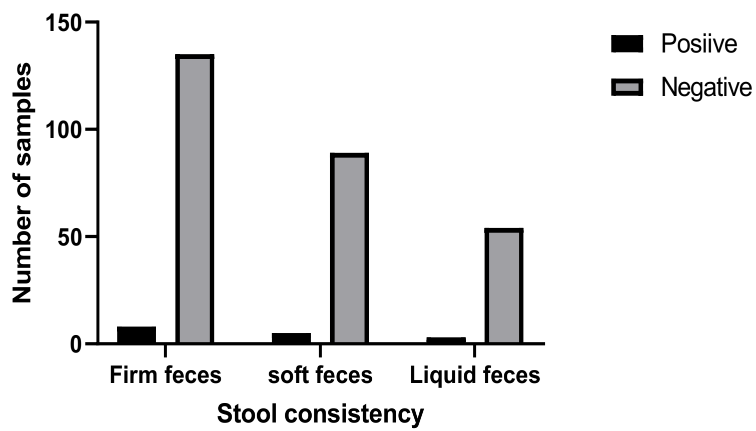


Fig. 7.4 Distribution of *E. moshkovskii* infection in pigs with their stool consistency.

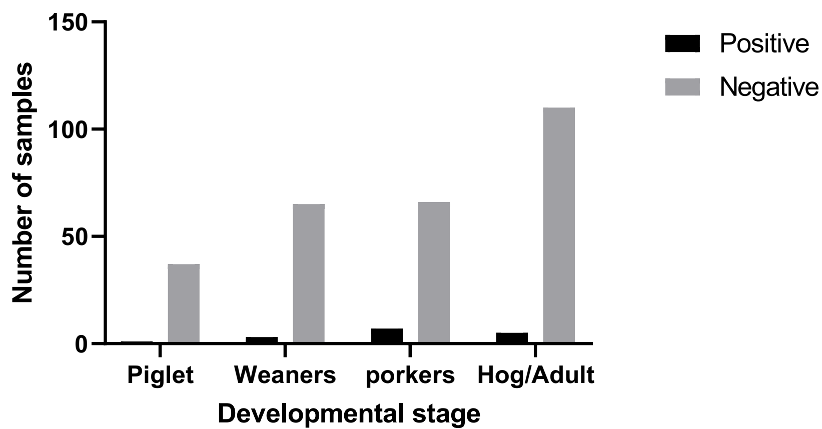


Fig. 7.5 Occurrence of *E. moshkovskii* infection in different developmental stages of pigs.

7.3.3. Rate of infection in different geographical location

A total of 88% (n/N= 14/16) of the specimens positive for *E. moshkovskii* were collected from the swine reared in the geographical location of Kolkata Leather Complex area (22.4984° N, 88.5172° E). Introduction of the infected individuals into a new demographic region may promote the establishment of new transmission cycle.

7.3.4. Nested PCR-RFLP of 18S rDNA locus

After the amplified PCR products were digested with restriction endonuclease MboII, we detected the presence of *Entamoeba* spp. The RFLP pattern for *E. moshkovskii* showed 214 bp and 44 bp fragments in this experiment. We observed that 100% (n = 16) of the obtained amplicons were digested by the MboII restriction enzyme and produced RFLP pattern for *E. moshkovskii*. Results of the nested PCR-RFLP for representative pig stool samples are shown in **Fig. 7.6**.

7.3.5. Phylogenetic analysis of *E. moshkovskii* isolates

Among the 16 positive samples of *E. moshkovskii*, 18S-rRNA sequences were obtained from 12 samples. Sequencing of the amplified PCR products demonstrated that they were of *E. moshkovskii* origin. The obtained sequence was deposited in NCBI GenBank database with accession number MW926950 and MZ357989–MZ357999 (18S rDNA). BLAST results showed that the seven representative sequences of all the 12 *E. moshkovskii* sequenced samples displayed 100% sequence similarity to *E. moshkovskii* Laredo strain (considered as a prototype) in NCBI Genbank (accession no. AF149906.1). DNA sequencing of four amplified products (MW926950, MZ357989, MZ357991, MZ357997 and MZ357989) showed that the sequence (258 bp) of this product was 99.46% identical to that of the corresponding reference sequence of *E. moshkovskii* (AF149906.1). While the remaining one sample (MZ357990) showed an identity of 99.22%. Only 1–2-bp nucleotide substitution (448T/G, 487G/T) was identified. Both the identified substitution corresponded to transversion (pyrimidine <=> purine) mutation. The constructed phylogenetic tree revealed that MW926950, MZ357989, MZ357991, MZ357997 and MZ357989 were the closest variants of the prototypes cluster (MZ357998–MZ357993), whereas MZ357990 was the most distant groups from prototype

cluster but they belonged to same species. MZ357990 shared a common clade with the closest variants of the prototypes cluster (**Fig. 7.7.**). The constructed Maximum Likelihood tree showed the similar results with PCR assay, i.e. they fitted in with *E. moshkovskii*. These results specify that the obtained *Entamoeba* isolates from swine stool in this report were *E. moshkovskii*.

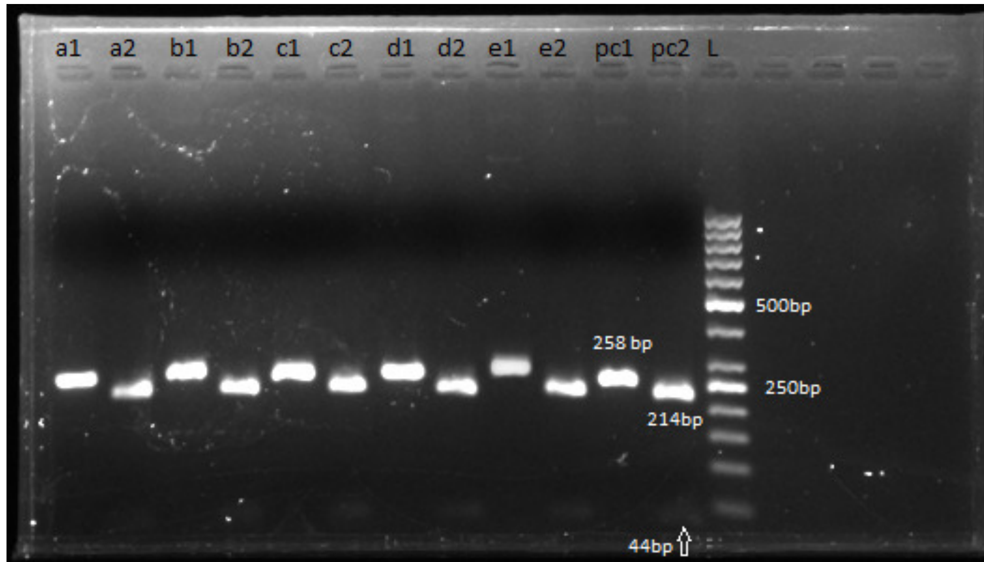


Fig 7.6. *Restriction* digestion of *Entamoeba moshkovskii*-specific nested 18S rDNA PCR product. a1, b1, c1, d1 and e1 are representative undigested nested PCR products of positive samples. a2, b2, c2, d2 and e2 are MboII digested PCR products. Pc1 is an undigested PCR product of positive control product. pc2 is MboII digested positive control PCR product.

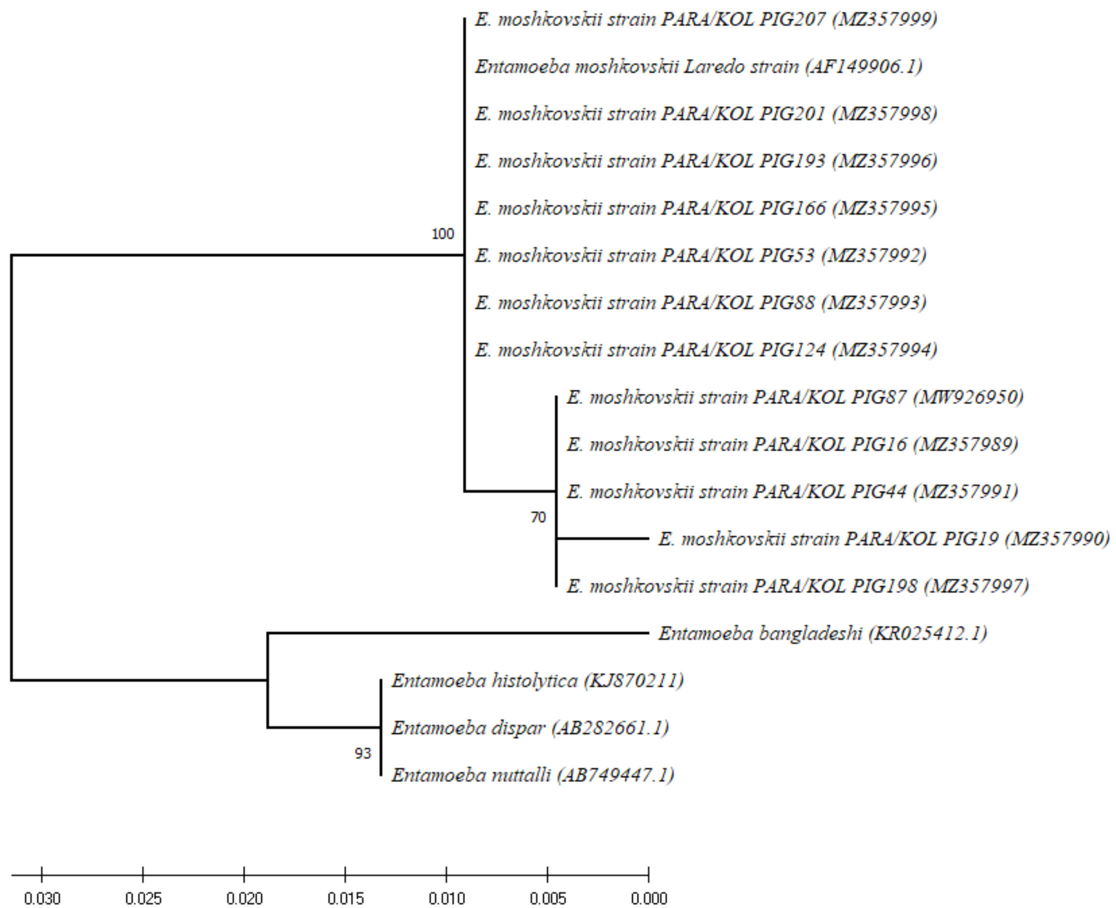


Fig 7.7. Phylogenetic analysis of *Entamoeba moshkovskii* (Tshalaia, 1941) isolates obtained from pig stool based on 18S rDNA locus from Kolkata and adjacent areas

7.4. Discussion

For the examination of *Entamoeba* spp. in stool samples (fresh or fixed), traditional microscopy has always been the most commonly used diagnostic tool. Although this strategy has a clear downside, which is that several species of *Entamoeba* are morphologically indistinguishable from each other via microscopy alone (e.g., *E. dispar*, a nonpathogenic species, is morphologically identical to *E. histolytica*). Therefore, to accurately identify the species/subtypes of *E. moshkovskii*, molecular tools including PCR and nucleotide sequencing had to be used (Verweij et al. 2003, Fotedar et al. 2007, Fotedar et al. 2007b, Khairnar et al. 2007, Delialioglu et al. 2008, Ngui et al. 2012).

The present study successfully identified *E. moshkovskii* from swine stool samples using molecular techniques. A moderately high prevalence rate of *E. moshkovskii* infection in pigs was recorded in this study. The prevalence of *E. suis* and *E. polecki* in pigs was reported in different studies to be from 3.7% to 91.7% (Ji et al. 2019). However, the occurrence of *E. moshkovskii* infection in pigs/farm animals has not been recorded. The present study showed that 87.8% of samples were positive for *Entamoeba* spp. and only 5.4% of samples contained *E. moshkovskii* DNA. Sequencing of amplified PCR products revealed the presence of *E. moshkovskii* in four novel genotypes while the remaining eight samples were similar to previously described genotypes reported in humans. We observed that the *E. moshkovskii* infection rate in pigs was not statistically associated with different variables like age, sex and stool consistency.

These results indicate that the infection occurred in a sporadic manner. Ingestion of polluted sources of water might be associated with their individual incidence. Investigation of the clinical importance of the infection in pigs was not performed in-depth for the study. More research studies are needed in order to get a better understanding of the transmission, zoonotic potential and epidemiology of *E. moshkovskii* in pigs. Genotyping of *E. moshkovskii* isolates from swine in different geographical areas could be performed for additional confirmation.

This is the first study that reports the occurrence of *E. moshkovskii* in farm animals to the best of our knowledge. Amoebae of the genus *Entamoeba* are well known as parasitic species but many of them are free-living as well. *E. moshkovskii* is an amphizoic amoeba that can occur in environmental or endozoic conditions (Clark and Diamond 2011). Until now, *E. moshkovskii* has been identified mostly in samples from treated wastewater used for agriculture, sewage and

human stool (Diamond and Bartgis 1970, Scaglia et al. 1893). Therefore, it can be transmitted between pigs by contact or ingestion of any unclean water sources. Although a previous study showed NHP (owl-faced monkey Cercopithecus hamlyni, Pocock, 1907; Javan lutung Trachypithecus auratus Geoffry, 1812 and Northern plains gray langur Semnopithecus entellus Dufresne, 1797) can be a novel host species for *E. moshkovskii* (see Levecke et al. 2010).

In general, a limited study has been conducted to explore the distribution of *E. moshkovskii* in animals. New host species for *E. moshkovskii* is yet to be identified. It will be significant in future studies to focus on zoonotic transmission and the pathogenic role of *E. moshkovskii* and to elucidate the host specificity of variants in a contest of amoebic infection. Our study was conducted in five different locations and *E. moshkovskii* occurred in two of them, which indicates that the distribution of *E. moshkovskii* is not uniform in these animals.

This study may boost research for a better understanding of the nature of infection of *E. moshkovskii*. Moreover, some recent findings support the pathogenicity of *E. moshkovskii* in humans and show that this amoeba may have a similar host range as *E. histolytica* (see Ali et al. 2003). The susceptibility of swine to *E. histolytica* was revealed in some experiments that indicated the role of swine as a reservoir for *E. moshkovskii*, which is closely related to *E. histolytica*. We speculate that the infection of swine with *E. moshkovskii* from their habitat was through the consumption of contaminated food or water and spread to other individuals via the faecal-oral route. The chances of transmission of the parasites from swine to humans cannot be ignored. Although this study was carried out in a fairly small number of samples, the high occurrence of *E. moshkovskii* in this study population indicates that pigs are true hosts for this parasite and the occurrence of *E. moshkovskii* in pigs warrants further attention. However, this study was conducted in a small geographical area. Studies in different geographical areas with an adequate number of samples should be considered to produce results among variables that might be significantly different.

This study provides the first data on the occurrence of *E. moshkovskii* infection in farmed pigs in eastern India. It shows that *E. moshkovskii* infection is prevalent in pigs. The overall prevalence of 5.4% of *E. moshkovskii* infection was reported in pigs. These results also suggest that pig might be a novel host species for *E. moshkovskii*. Thus, more attention should be given to evaluating the chance of transmission of *E. moshkovskii* from natural reservoirs to humans.

The life cycle of commensal *Entamoeba* is characterized by two distinct stages: the dormant cyst and the active trophozoite stage (Faust and Guillen, 2012). Mature cysts, serving as the infectious form of the parasite, are commonly detected in faecal samples. On the other hand, trophozoites, representing the adult trophic form, are typically found within the intestines of the host, with an occasional presence in the diarrheal stool. In previous chapters, we noted a significant prevalence of *E. moshkovskii* infection among patients with diarrhoea. Additionally, we observed that the rate of human infection with *E. moshkovskii* tends to increase during the post-monsoon season. This is attributed to the spreading of dormant cysts of *E. moshkovskii* during the monsoon period, which eventually reaches its peak in the post-monsoon season.

The spread of *E. moshkovskii* cysts can occur through two main pathways. Firstly, it can be transmitted from human to human through the faecal-oral route. Secondly, the cysts released by pigs can be transmitted to humans, leading to infection through a zoonotic transmission pathway. As previously mentioned, *E. moshkovskii* is capable of infecting both humans and pigs, and pigs can acquire the infection either directly from the environment or from other infected pigs through the faecal-oral route. Another route of *E. moshkovskii* infection is through the reverse zoonotic pathway, wherein the cysts released by E. *moshkovskii* infected pigs can lead to human infection. In this scenario, the cysts originating from infected pigs can be transmitted to humans, causing the reverse zoonotic transmission of *E. moshkovskii* (see **Fig 7.8**).

Another noteworthy observation is that during the summer season in West Bengal, there is a significant increase in the infection rate of *E. moshkovskii*. This can be attributed to the higher temperatures and the drying up of water bodies from winter to summer. These environmental conditions exert stress on the free-living amoeba *E. moshkovskii* trophozoites that inhabit water bodies and sewage. Environmental stresses, such as elevated temperatures, water bodies drying up, and pH changes, may facilitate the process of encystation in free-living strains of *E. moshkovskii*. Following encystation, these cysts can readily infect humans and animals such as pigs. This suggests that the environmental stress during the summer season plays a role in facilitating the transmission of *E. moshkovskii* to both humans and animals. Following infection in either humans or animals like pigs, the infection can readily propagate via the faecal-oral route, involving zoonotic or reverse zoonotic transmission pathways (see **Fig 7.8**).

The suggested transmission pathway was proposed considering the epidemiological data on the prevalence of *E. moshkovskii* in humans and animals, as well as its seasonal patterns of

infection. However, conducting thorough research is essential to accurately delineate the life cycle and transmission route of *E. moshkovskii* infection. By unravelling the transmission pathway of *E. moshkovskii*, it becomes possible to enhance prevention and control measures against diarrheal incidents caused by this pathogen.

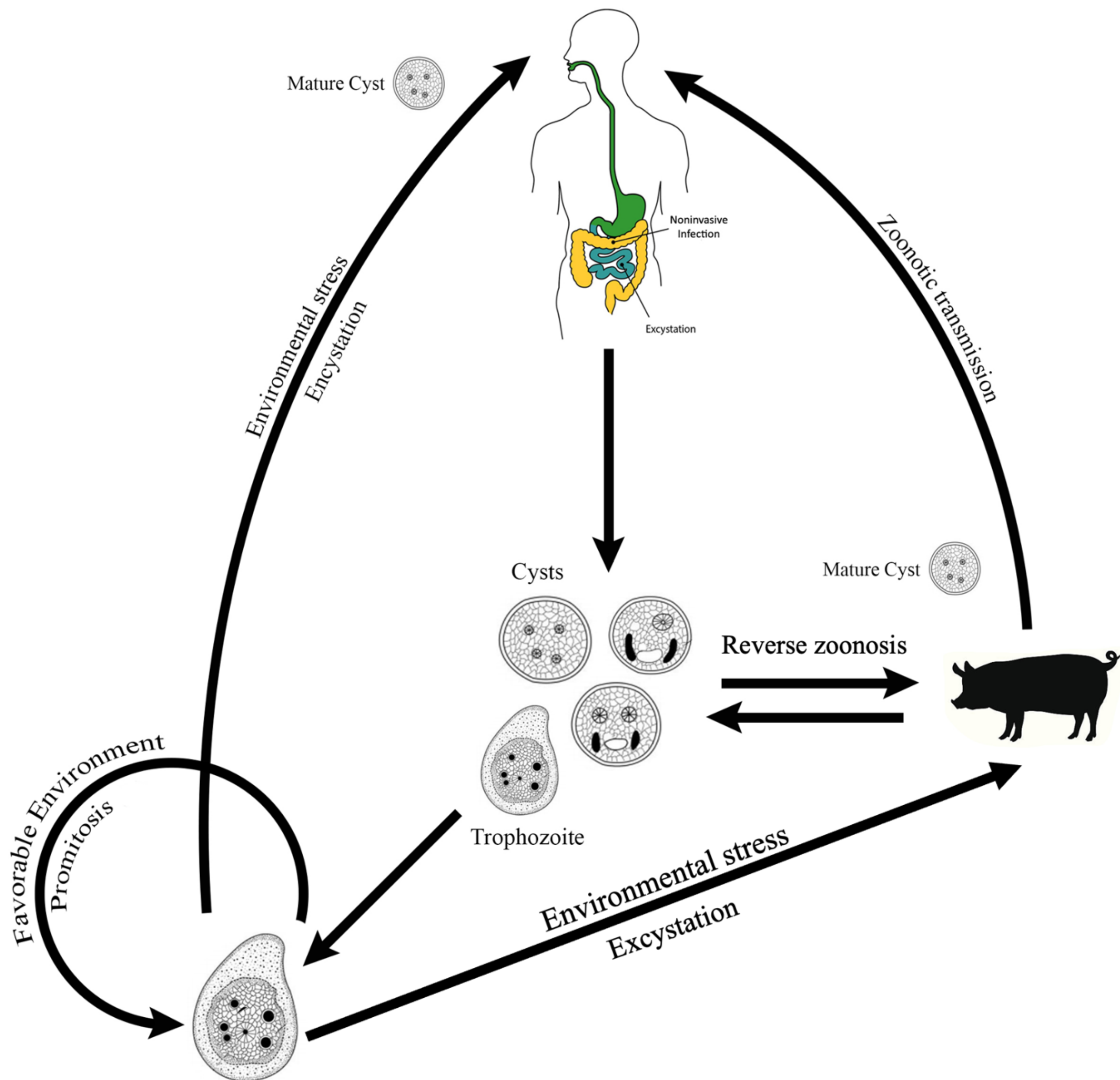


Fig. 7.8 Illustration depicting the proposed life cycle and transmission pathway of *E. moshkovskii* infection.

7.5. Conclusion

The ability of *E. moshkovskii* to grow in the environment and to adapt to adverse conditions as well as able to reside in animal intestine such as pigs suggest that the risk of infection should be higher than the risk of *E. histolytica* or *E. dispar* infections. The significant prevalence of *E. moshkovskii* in pig populations suggests that pigs serve as true hosts for this parasite, highlighting the need for increased attention. In the preceding chapter, the study reported a high rate of diarrhoea caused by *E. moshkovskii* infection. The findings of this chapter raise concerns about the potential transmission of *E. moshkovskii* from pigs to humans, indicating a possible zoonotic threat The study suggests the implementation of effective control measures to prevent the zoonotic transmission of *E. moshkovskii* infection among individuals handling animals. Furthermore, it recommends conducting a comprehensive investigation in regions where pig farming is prevalent, with a particular focus on pig handlers, to accurately evaluate the potential for zoonotic transmission of *E. moshkovskii*.

CHAPTER VIII

GENOTYPING OF *E. HISTOLYTICA* ISOLATES BASED ON DIFFERENT POLYMORPHIC LOCI

Genotyping of *E. histolytica* isolates based on different polymorphic loci

8.1. Background

The analysis of epidemiological data in the previous chapters has uncovered the emergence of *E. moshkovskii* as an enteric pathogen associated with the prevalence of diarrhoea in our study area. While *E. moshkovskii* plays a significant role in amoebic infections within our study samples, it is important to note that *E. histolytica* remains the sole invasive enteric parasite identified thus far. We noticed a substantial prevalence of *E. histolytica* infection in our hospital-based surveillance study. Conducting genotyping studies on *E. histolytica* in more isolates is crucial for obtaining valuable data to implement effective prevention and control measures against *E. histolytica* infections.

Amoebiasis, caused by the parasite *E. histolytica*, is considered one of the most serious gastrointestinal infections in humans (Das et al. 2014). It is the third leading cause of death due to parasite infection after schistosomiasis and malaria (Singh and Galhotra, 2014). The disease can spread via the faecal-oral route and is highly endemic in tropical and subtropical countries with an average socioeconomic status and poor public health (Guillén, 2023; Preet et al. 2011). However, only one out of five individuals infected with *E. histolytica* develops intestinal or extra-intestinal diseases, and the remaining 80% of infections are asymptomatic (Yanagawa et al. 2020). The specific determinants for this differential outcome of infection are yet to be fully understood, although host defence mechanisms and pathogen virulence are believed to be primary controlling factors. Some studies have reported that host immune responses, such as anti-inflammatory cytokines and interleukins, play a critical role in preventing or interrupting tissue invasion, and specific human leukocyte antigen/HLA class-II alleles have also been found to be associated with susceptibility to *Entamoeba histolytica* infection (Escueta-de Cadiz et al. 2010). However, it is still uncertain what other factors in the relationship between host and parasite define the outcome of the disease, especially with regard to the regulation of host immune responses to the pathogenic *E. histolytica*.

On the other hand, DNA typing of various polymorphic genetic loci such as serine-rich *E. histolytica* protein (SREHP), Chitinase, and tRNA-linked STR loci (A-L, N-K2, R-R, S-Q, and S^{TGA}-D), have been performed to try to find a possible link between a parasite genotype and the outcome of infection for the last two decades and several reports have correlated specific genotypes with virulent (or avirulent) phenotypes (Ali et al. 2007; Das et al. 2013, Escueta-de Cadiz et al. 2009; Jaiswal et al. 2014; Haghighi et al. 2002). But, one major limitation of this

genotyping strategy is that polymorphism of the loci mentioned above is highly divergent, and it characterises several genotypes even in a limited geographic area (Haghighi et al. 2002). As the number of genotypes was too many, such a correlation between individual parasite genotypes and different infection outcomes became difficult for evaluating the clinical utility in some studies (Jaiswal et al. 2014). Therefore characterization of additional isolates using additional genetic markers is recommended to unveil the mystery between genetic polymorphisms in *Entamoeba histolytica* and clinical phenotypes of amoebiasis.
Among the many molecular markers, SREHP and tRNA-linked array N-K2 are the two highly polymorphic loci most important in genotyping studies of *E. histolytica* infection (Ali et al. 2008; Zermeño et al. 2013). The protein serine-rich protein of *E. histolytica*, SREHP is a prominent surface antigen that contains repetitive sequences of closely related dodecapeptides and octapeptides (Köhler and Tannich 1993; Stanley et al. 1990). A study in Limpopo province showed that specific SREHP genotypes might be associated with amoebic intestinal symptoms (Samie et al. 2008). Ayeh-Kumi *et al.* reported that SREHP genotypes observed in liver abscess patients differed from those with amoebic colitis and dysentery in Bangladesh (Ayeh-Kumi et al. 2001). However, other studies did not support this premise (Haghighi et al. 2003). Das et al. observed a significant association between parasite genotypes and the outcome of amoebic infection from India based on the widely used repetitive marker SREHP. Still, the exact genetic traits determining the virulence capacity of the parasite were not specified. Among tRNA-linked STR loci, N-K2 is the most polymorphic genetic marker (Das et al. 2014). Many unique N-K2 genotypes were reported from intestinal *E. histolytica* infection in asymptomatic carriers and symptomatic diarrhoeal patients. This locus exhibited a highly significant difference in the distribution of genotypes between the asymptomatic and the liver abscess among *E. histolytica* patient groups (Ali et al. 2007).

Loci 1–2 and 5–6 are the two tRNA-linked polymorphic loci containing internal tandem repeats (Zaki and Clark, 2000). These two loci were first isolated and characterized by Zaki and Clark 2000 (Zaki and Clark, 2000). The locus 1-2 is now designated as tRNA linked D-A locus (Escueta-de Cadiz et al. 2009). However, we preferred the term locus 1-2 throughout the study. Loci 1–2 and 5–6 are multicopy and may be arranged in tandem arrays. DNA sequencing of the two loci revealed that both contained 6 to 21 copies of 8 to 16 nucleotide repeat units and alterations in the number of repeat units associated with observed size variation in PCR products (Haghighi et al. 2002). Nucleotide sequences of the two loci of *E. histolytica* are significantly different from *E. dispar* in both repeats and flanking regions (Zaki et al. 2002a;

Pattanawong et al. 2021).Moreover, many distinct isolates of *E. histolytica* with slight differences in PCR product size were reported in many cases. Haghighi et al. 2002 conducted a nucleotide polymorphism study of the two loci in different *E. histolytica* isolates from a limited geographic area and did not observe any association between genotypes and clinical manifestation (Haghighi et al. 2002). These two crucial polymorphic loci can be used in genotyping studies of *E. histolytica* infection. They could be beneficial for the population structure of *E. histolytica* and for exploring the molecular epidemiology of amebiasis (Haghighi et al. 2002; Haghighi et al. 2003). But a genotyping analysis using these two loci is limited.

The abovementioned loci are crucial genetic markers in the genotyping study of *E. histolytica.* More studies using these loci are required to evaluate the association between parasite genotypes and the clinical presentation of amoebiasis.

Due to its low resolution, single polymorphic locus genotyping cannot be used to identify all genotypes of *E. histolytica*. Employing multiple loci, we have focused on developing a strain typing strategy that is optimal and provides high resolution (Das et al. 2014).

In this study, genotyping of *E. histolytica* clinical isolates obtained from diarrheal, non-diarrheal and liver abscess samples was performed using one polymorphic coding gene viz. SREHP and three polymorphic non-coding loci viz. tRNA linked array N-K2, loci 1–2 and 5–6. We were targeting a multilocus STR polymorphism investigation to decipher the genetic background of *E. histolytica* isolates sporadically becoming diarrhoeagenic or causing an amoebic liver abscess (ALA). We also investigated the genetic pattern and the diversity of *E. histolytica* isolates obtained from asymptomatic infected individuals. Therefore, the present study might help us understand the association between the parasite genotypes and outcomes of amoebic infection.

8.2. Methodology

8.2.1. Sample collection

A total of 3258 diarrhoeal and 2265 non-diarrhoeal were collected and screened in this study. This study included a total of 12 samples of amoebic liver abscesses. The diarrhoeal samples were obtained from patients admitted to Infectious Disease & Beliaghata General Hospital Hospital, Kolkata, India, with sole diarrheal complaints, and non-diarrhoeal samples were

obtained from an ongoing field project investigating Helminth parasite burden in eastern India. The study population mostly belonged to lower socioeconomic communities that government healthcare facilities supported. This study was conducted from March 2017 to February 2022. Liver aspirates of amoebic liver abscesses were collected personally from various hospitals in Kolkata as part of a collaborative research endeavour. All samples were immediately sent to laboratories for processing within 4 hours of collection. The institutional ethical committee of ICMR-NICED approved the protocol of this study. Informed consent was taken from the participants, and samples were coded anonymously *to preserve* the confidentiality of the study *participants.*

8.2.2. Identification of *E. histolytica*

The parasites were primarily identified by microscopy in both saline wet mounts and Lugol's iodine wet mounts. Total genomic DNA was extracted directly from each stool specimen found positive for *Entamoeba* spp through microscopic examination, using STOOL DNA Minikit (QIAGEN, USA) as per the manufacturer's protocol. The presence of *E. histolytica* in stool samples was further confirmed by molecular identification using PCR amplification of the SSU rDNA locus. The following primer pair was used in the PCR amplification: EH1: GTACAAAATGGCCAATTCATTCAATG and EH2: ACTACCAACTGATTGATAGATCAG (Mukherjee et al. 2009). The expected band size is 135 bp. The amplification was performed in a 50µl reaction mixture using TaKaRa r-Taq polymerase (Takara). The PCR amplification mixture contained an amount of 3µl stool DNA. The PCR temperature cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by a total of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds and polymerization at 72°C for 25 seconds and final extension at 72°C for 7 minutes. Amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualised under UV-transilluminator.

8.2.3. Genotyping of *E. histolytica* isolates using PCR and DNA sequencing

Fifty-four samples positive by PCR were selected to amplify one polymorphic coding gene SREHP and three polymorphic non-coding loci viz. tRNA linked array N-K2, loci 1–2 and 5–6. In all cases, the amplification was carried out in a 50µl reaction mixture containing 5U TaKaRa Ex-Taq polymerase, 1X PCR buffer, 0.2 µM of each forward and reverse primer and 3 µl of stool DNA samples at 50 ng/µl concentration. Reactions were carried out in a thermal cycler PCR system (Applied Biosystem). The PCR cycles were started with an initial

denaturation step at 94°C for 5 minutes, followed by 35 cycles of amplification consisting of denaturation at 94ºC for 40 seconds, annealing at 53ºC (Loci 1-2) and 56ºC (Loci 5-6, SREHP and NK2) for 35 seconds and polymerization at 72ºC for 45 seconds and final extension at 72ºC for 7 minutes. Primer sequences and annealing temperatures employed are provided in Table 1. The amplified PCR products were separated by agarose gel (Seakem® LE Agarose, Lonza) electrophoresis and observed under UV-transilluminator after 0.5µm/ml ethidium bromide staining.

The PCR products of the expected sizes, each of the bands, whether single or double, were individually excised from an agarose gel after electrophoresis. Subsequently, the extracted bands were purified using a Roche Gel extraction kit following the manufacturer's protocols. Their yield was rechecked by gel electrophoresis. The purified PCR products were sequenced directly using the individual amplification primers in both directions with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) and then analysed on an ABI3730 sequencer.

8.2.4. Sequence analysis

The obtained DNA sequences were aligned employing the clustalW multiple sequence alignment program of GenomeNet Bioinformatics resources and edited manually. The process of identifying STRs involved both manual searches for known repeat units in the obtained nucleotide sequences and using the Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) tool to discover novel STRs. For loci 1-2 and 5-6, we conducted a manual search for repeat units previously reported by Zaki and Clark, 2001 and Haghighi et al. 2002. As for the SREHP and NK2 loci, we performed manual searches based on the findings reported by Das et al. 2016 and Haghighi et al. 2003. Each repeat unique was denoted with an individual pattern fill. The newly identified sequence types obtained in this study were assigned with alphanumerical codes followed by country names in a subscript manner—alphanumeric codes ascribed newly identified repeat patterns. Then the STR sequence patterns found from four loci were combined to assign a genotype. The obtained genotypes were also assigned alphanumerical codes beginning with the alphabet “I” to specify their Indian origin. The nucleotide sequences reported in this study have been deposited to NCBI GenBank. Because all the loci are highly polymorphic and commonly used to assess population genetic structure, in *Entamoeba histolytica* isolates, we inferred relationships among haplotypes by constructing a Median

Joining haplotype network in PopART v1.7. The isolates were colour-coded according to their disease outcomes to derive relationships with sequence data.

8.2.5. *Statistical analysis*

GraphPad Prism 9, CA, USA was used for analysing the categorical data. The relationship between the genotypes/repeat patterns and clinical phenotypes was measured using Fisher's exact test. A p-value of < 0.05 was considered statistically significant in all cases.

Primer	Primer sequence (5'-3')	Annealing Temperature °C
R1[#]	CTGGTTAGTATCTTCGCCTGT	53°C
R2[#]	CTTACACCCCCATTAACAAT	
R5A[#]	CTAAAGCCCCCTTCTTCTATAATT	56°C
R6A[#]	GTGCTAATAACGCCAGGGTC	
NK2H5[*]	GAAGCGTCTTTTTACTATTAGTG	56°C
NK2H3[*]	GGCGTATTTTTAGAATAGGATAAG	
SREHP_F	TGAGTGGAACATAATATCGATGAAA	56°C
SREHP_R	CAATGAATGGACTTGATGCAG	

[*]Ali et al. 2005
[#] Zaki and Clark, 2001

Table 8.1 Oligonucleotide primers for screening of *E. histolytica* in the studied sample.

8.3. Results

Of the 3258 diarrhoeal samples analysed, 0.83% (n/N= 27/3258, 95% CI 0.0056 to 0.0121) were found to be positive for *E. histolytica* through PCR testing. Out of 2226 asymptomatic individuals, 0.66% (n/N= 15/2265, 95% CI 0.0039 to 0.0110) of them tested positive for *E. histolytica*. In all cases of amoebic liver abscess (ALA), PCR testing confirmed 100% positivity for *E. histolytica* (n/N= 12/12, 95% CI 0.7180 to 1.0000). All positive samples were subjected to further amplification using targeted polymorphic loci for genotyping. The amplified PCR products of the targeted loci revealed significant polymorphism, which depended on the repeat types, numbers, and orientation present in the isolates being studied.

8.3.1. Polymorphism in length and nucleotide sequences

We have successfully sequenced all relevant loci for 42 DNA samples. However, for the remaining 12 samples, we only amplified loci 1-2, loci 1-5, locus 6, and the tRNA-linked array N-K2 locus. The PCR fragments showed notable polymorphism in size and their number of bands at SREHP and 5-6 loci. 1-2 loci and NK2 loci showed only variations in PCR product sizes (**Fig. 8.1**). Consistent with previous findings, none of the isolates had more than two gel electrophoresis bands in SREHP and loci 5-6. Further DNA sequencing revealed more pronounced polymorphisms than those displayed by agarose gel electrophoresis. Similar-sized PCR products showed differences in DNA sequences with distinct repeat patterns in many isolates. NK2 and SREHP loci were highly polymorphic, whereas loci 1-2 and loci 5-6 were moderately polymorphic. Upon DNA sequencing, the PCR product sizes for locus 1-2 ranged from 387bp to 438bp, for loci 5-6 from 258bp to 516bp, for locus NK2 from 519bp to 665bp, and for loci SREHP from 633bp to 734bp.

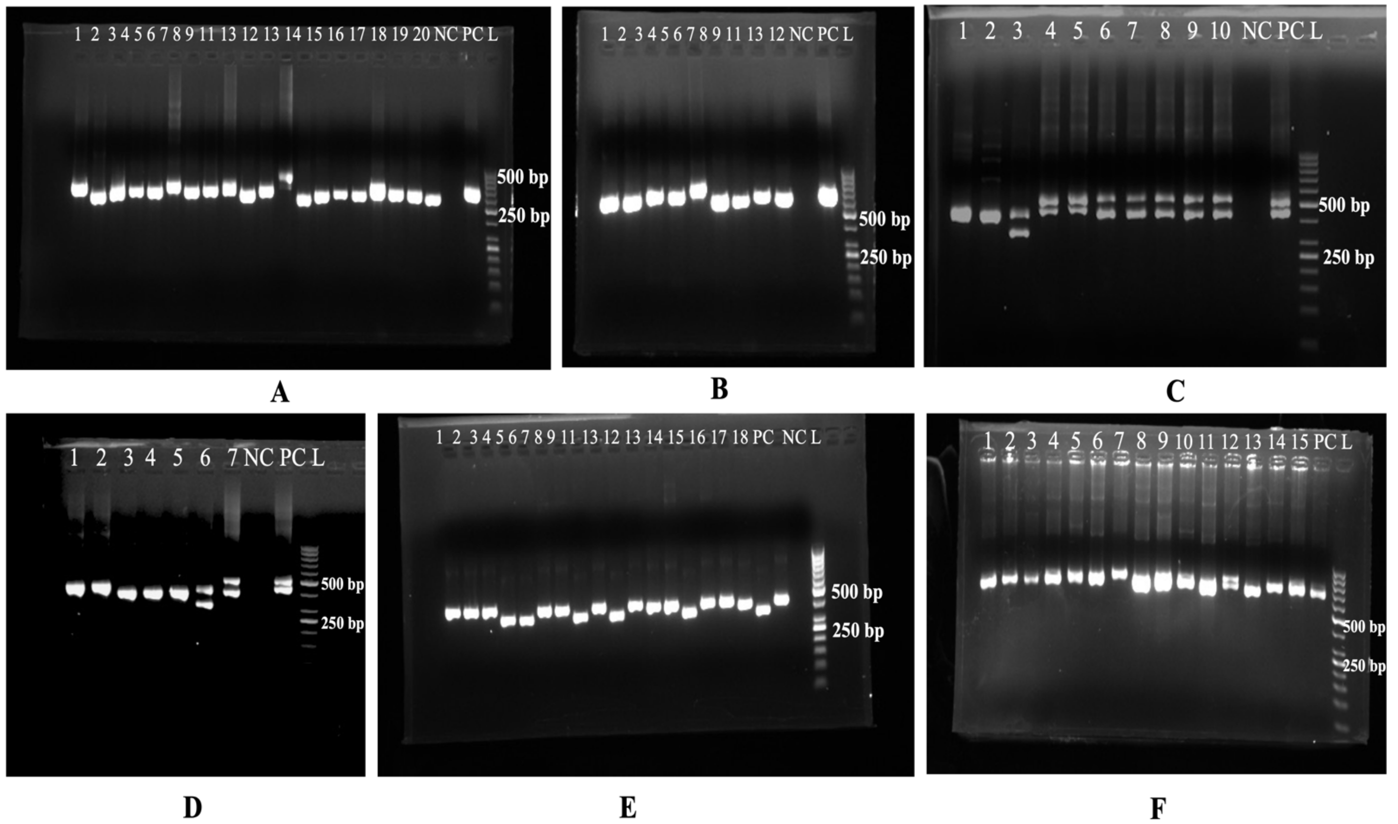


Fig. 8.1 The figure shows the representative amplified PCR products of several studied STR loci of *E. histolytica*. A and B display the PCR products for the N-K2 locus, while C and D show the products for the locus 5-6. E displays the product for locus 1-2, and F shows the product for the SREHP Locus. NC-Negative control, PC-Positive Control, L-DNA ladder.

8.3.2. Polymorphism in tRNA linked array N-K2 locus

We have sequenced the individual amplified PCR products of N-K2 loci to understand the nature of the polymorphisms among the studied isolates. The nucleotide polymorphisms shown by DNA sequencing were more prominent than gel electrophoresis. This locus contained 1-27 copies of four 8-nucleotide repeat units, consistent with previous studies (Das et al. 2014). The observed repeat units were TTAGTAAT, TTAGTATT, CACTTATC & CACTTATA. Sequencing of N-K2 loci showed a complex polymorphism between the isolates concerning their location, length and number of nucleotide repeat units. Overall, we revealed a high level of polymorphism within the N-K2 loci among our 54 study isolates. A total of 20 different repeat patterns were observed; all were not previously described. The newly identified patterns were assigned as K1-K20 ((**Fig. 8.2**). K15 was the most dominant (12.96%, n/N=7/54) repeat pattern (**Fig. 8.3**). The distribution of the repeat pattern in different disease outcomes groups is presented in table **no 2**. Newly identified sequences of N-K2 locus had been deposited to GenBank with accession numbers OQ810098 - OQ810151.

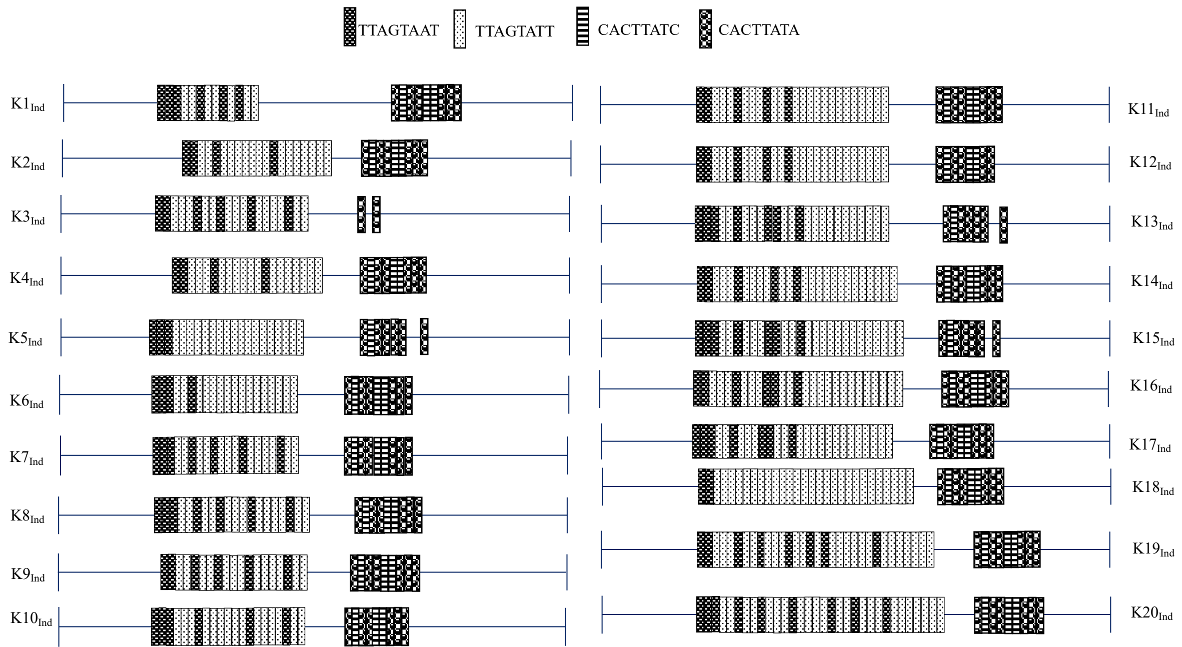


Fig. 8.2 Diagram illustrating the repeat patterns of the NK2 locus, as determined from the nucleotide sequences of the isolates analyzed in this study. The repeat units are represented by rectangles filled with distinct designs, and 20 novel repeat patterns were identified and labelled as $K1_{Ind}$ to $K20_{Ind}$.

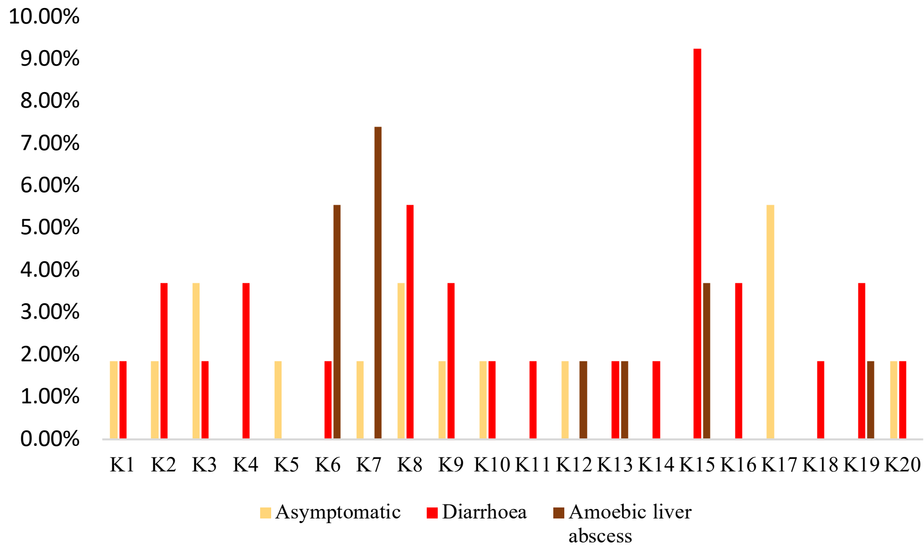


Fig. 8.3 This figure illustrates the distribution of N-K2 genotypes obtained from *E. histolytica* isolates of different disease outcome groups where each genotype is depicted by a unique colour. K1-K20 are the abbreviation for $K1_{Ind}$ -$K20_{Ind}$.

8.3.3. Polymorphism in locus 1-2

Locus 1-2 was moderately polymorphic in our study isolates. Based on the combination pattern of constructing units, nine allelic variations were obtained, out of which six were newly identified and assigned $L1_{Ind}$- $L6_{Ind}$, and the other three (B, E & F types), were previously described by Haghighi et al. 2002. F type was the dominant (22.22%, n/N=12/54) variant in the studied population. This locus contained 1-10 copies of eight repeat units, each having 8-12 nucleotides (**Fig. 8.4**). We identified a previously unreported 12-nucleotide repeat unit (TACTCTTTTTAC) present in B, E, and F types, as well as in other newly reported sequence types. Notably, this 12-nucleotide repeat unit differs from the 13-nucleotide repeat unit (CTTTTTACTACTC) reported by Haghighi et al. 2002 and Zaki and Clark, 2001. The distinct 12-nucleotide repeat unit was found in a similar position to the 13-nucleotide repeat unit on locus 1-2 within the analyzed sequences. $L4_{Ind}$ and $L6_{Ind}$ types/patterns obtained in this study were exclusive for the diarrhoeal (D) group (**Fig. 8.5**). Repeat pattern information of this locus in different disease outcome groups has been shown in **table no 2**. The newly identified pattern with unique sequence repeats was submitted to NCBI with accession numbers OQ810205 - OQ810258.

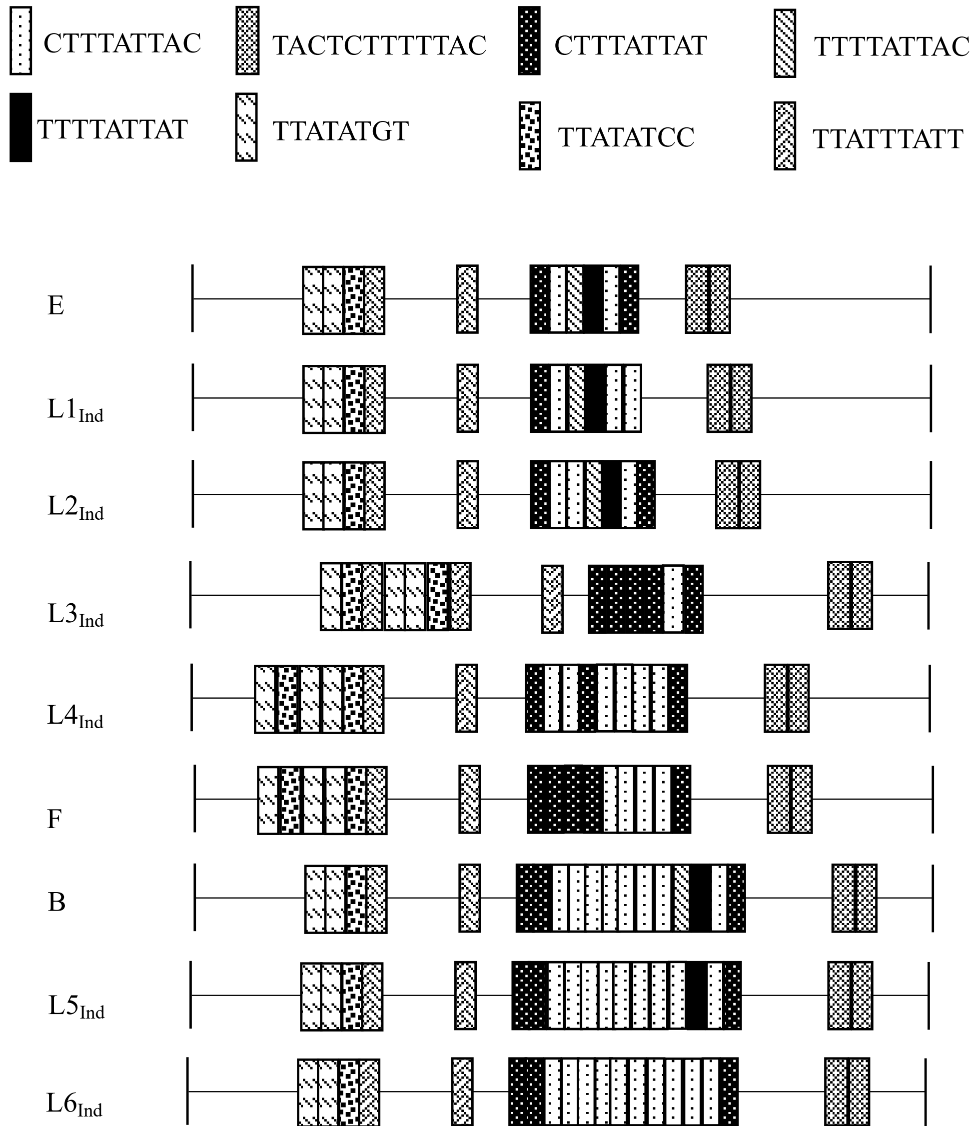


Fig. 8.4 Figure depicting the repeat patterns of locus 1-2, as identified from the nucleotide sequences of the isolates analyzed in this study. The repeat units are represented by rectangles filled with unique designs. A total of 9 repeat patterns were observed and labelled as $L1_{Ind}$ to $L6_{Ind}$.

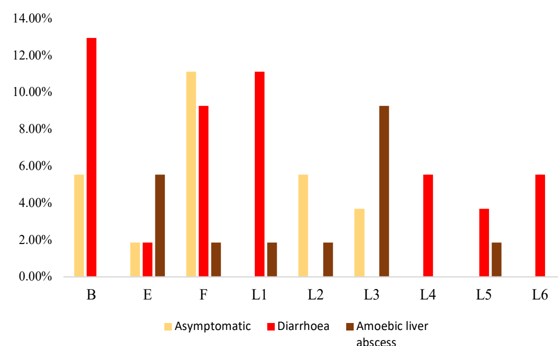


Fig. 8.5 The presented figure displays the distribution of various haplotypes of *E. histolytica* isolates in different disease outcome groups based on locus 1-2. Each haplotype is distinguished using a unique colour.

8.3.4. Polymorphism in locus 5-6

Locus 5-6 showed either single or double bands of amplified PCR products depending upon the isolates, which is consistent with the previous reports. Sequencing of the individual bands showed ten different types of PCR products with distinct repeat patterns, out of which seven were newly found and assigned as $Q1_{Ind}$ -$Q7_{Ind}$, and the remaining three (A5, A6 & A7 type) were reported by Haghighi et al. 2002 (**Fig. 8.6**). The 12-nucleotide repeat unit (GTATGTCTCTAT) reported by Zaki and Clark, 2001 was notably absent in our study isolates. The polymorphism of the locus primarily results from variations in 16-nucleotide repeat units (TATGTATATTTCTATG), occurring with 5 to 9 repeats. Additionally, during our investigation, we identified a new 50-nucleotide repeat unit (AAAAACAATGCTCTCAGCAGGTTTCGAACCTGCGACCCTGGCGTTATTAG; occurring with 0 to 2 repeats) that also contributes to the observed polymorphism. In this study, we also recorded a unique DNA sequence in this locus that contained a newly identified 16-base tandemly arranged repeat unit (TACATAGAAATATACA; 8 repeats) assigned as a $Q10_{Ind}$ pattern (**Fig. 8.6**). We did not find any commonly reported repeat unit in the $Q10_{Ind}$ pattern of locus 5-6. Furthermore, amplification of the locus results in two bands in 46.30% (n/N=25/54) of the isolates. This result suggests polymorphism between the homologous loci on allelic chromosomes of the *E. histolytica* genome. Alternatively, the observed multiple bands could be interpreted by the presence of numerous copies of the tandem repeat sequences at various locations in the *Entamoeba* genome, each with a specific size. The frequency distribution of different haplotypes among distinct disease outcome groups has been classified based on their respective repeat patterns as presented in in **Fig. 8.7**. Repeat pattern information of locus 5-6 in study isolates has been provided in **table 2.** Representative nucleotide sequences of locus 5-6 have been submitted to NCBI GenBank with accession numbers OQ810259 - OQ810337.

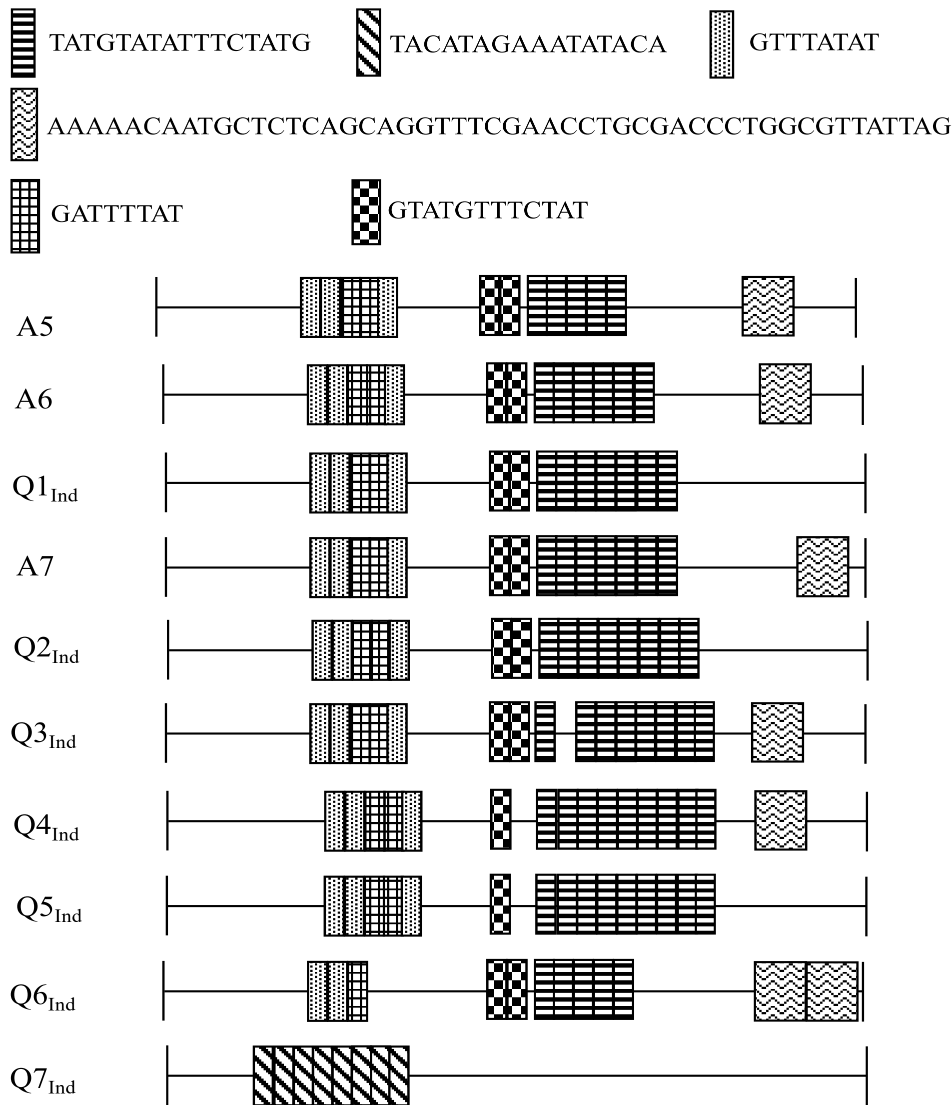


Fig. 8.6 Figure depicting the repeat patterns of locus 5-6, as identified from the nucleotide sequences of the isolates analyzed in this study. The repeat units are represented by rectangles filled with unique designs. A total of 10 repeat patterns were observed and labelled as $Q1_{Ind}$ – $Q7_{Ind}$.

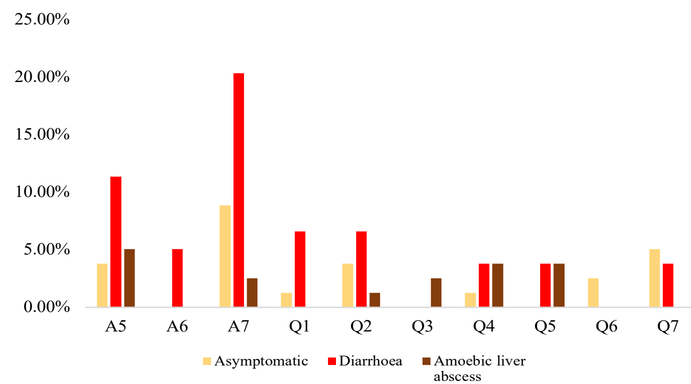


Fig. 8.7 This figure displays the distribution of different haplotypes of *E. histolytica* in various disease outcome groups based on locus 5-6. Each genotype is distinguished using a unique colour. Q1-Q7 are the abbreviation for $Q1_{Ind}$ -$Q7_{Ind}$.

8.3.5. Polymorphism in SREHP

The SREHP gene was successfully amplified from 48 clinical samples. In most of the samples, amplification results in a single band. However, in five samples, SREHP was amplified in two bands. Previous studies reported a single copy SREHP gene, indicating that the length differences are allelic variations (Zaki and Clark, 2001). The SREHP gene was highly polymorphic. We found 15 different repeat patterns, newly identified and assigned as $S1_{Ind}$ to $S15_{Ind}$ (**Fig. 8.8**). Repeat pattern information among different disease outcomes has been provided in **Table no 2.** A 50-nucleotide repeat unit (CTCAGCAAAACCAGAATCAAGTTCAAATGAAGATAATGAAGATGATGAAG

; 0-2 repeats) in SREHP gene has been identified in this study which has not been previously reported. We have also found a unique 16-nucleotide unit (GAATCAAGCTCAAGTGATAAAAT) in sequences of $S11_{Ind}$ and $S13_{Ind}$ patterns, which was not described in earlier studies. Although we obtained a single copy of this unit, which matches 87.5% of another unit (GAATCAAGCTCAAGTGATAAACCA), we can not rule it out as a sequencing artefact because it has been found in eight samples. The majority of obtained repeat patterns were non-exclusive for particular disease outcome groups except $S8_{Ind}$ and $S3_{Ind}$ /$S4_{Ind}$. However, we did not observe any significant association of $S8_{Ind}$ and $S3_{Ind}$ /$S4_{Ind}$ patterns with their occurrence in diarrheal cases. $S6_{Ind}$ was the rare pattern with a single event in the diarrhoeal disease outcome group (**Fig. 8.9**). Moreover, double bands were detected in 10.20% (n/N= 5/49) of the PCR-amplified products of the SREHP locus. Earlier research also demonstrated similar findings. In 2001, Zaki and Clark conducted a Southern blotting technique, which indicated that the SREHP locus corresponds to a single-copy gene (Zaki and Clark, 2001). Therefore, the presence of multiple bands produced from the SREHP gene could result from polymorphic variations among homologous loci on different alleles of the chromosomes. Another possible reason for the presence of multiple bands could be that an individual is infected with different strains of *E. histolytica* (Ayeh-Kumi et al. 2001). The accession numbers OQ810152 - OQ810204 have been assigned by NCBI GenBank to the representative nucleotide sequences submitted for locus SREHP.

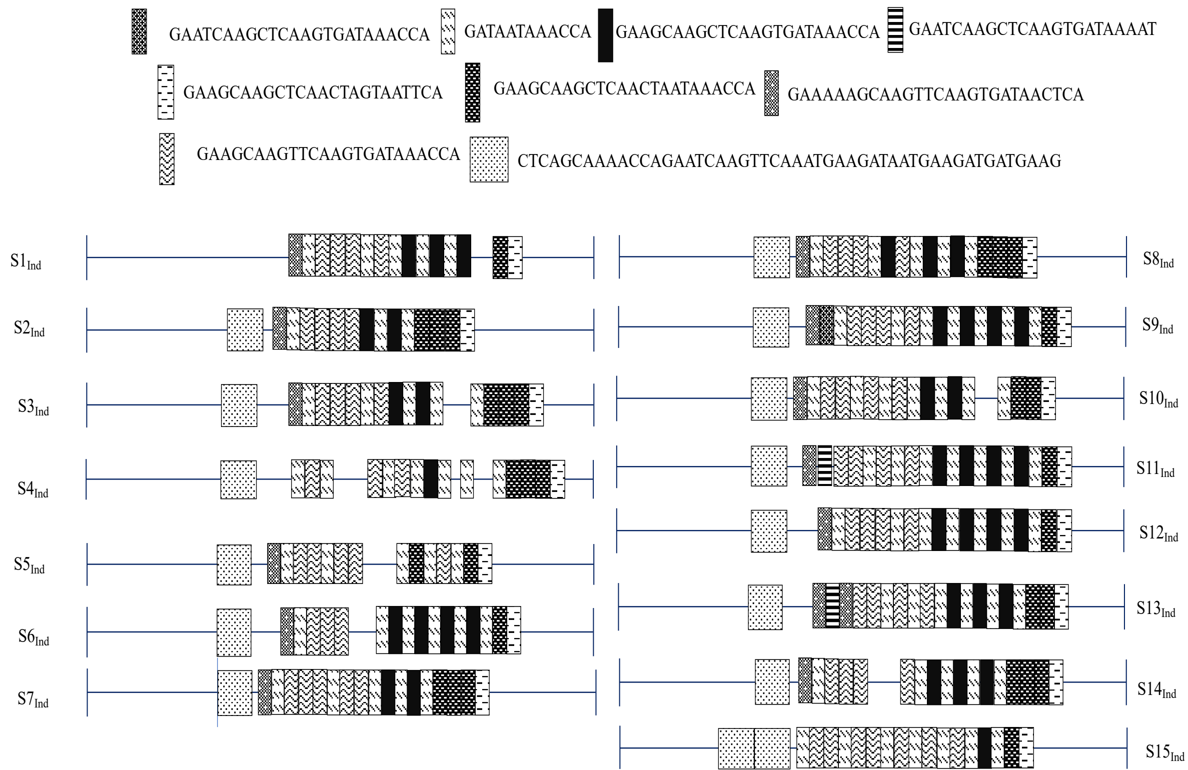


Fig. 8.8 Figure depicting the repeat patterns of SREHP, as identified from the nucleotide sequences of the isolates analyzed in this study. The repeat units are represented by rectangles filled with unique designs. The repeat units are represented by rectangles filled with distinct designs, and 15 novel repeat patterns were identified and labelled as $S1_{Ind}$ to $S15_{Ind}$.

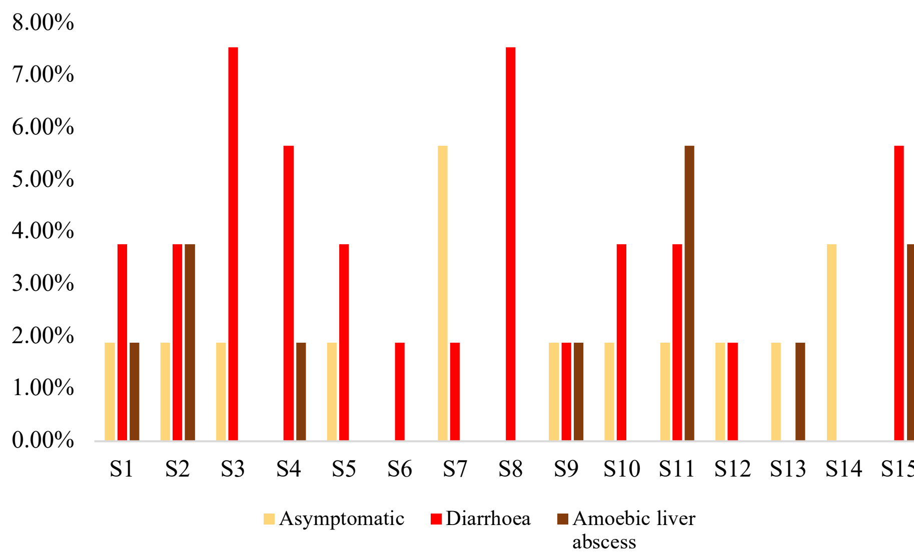


Fig. 8.9 This figure displays the distribution of different haplotypes of *E. histolytica* in various disease outcome groups based on SREHP. Each genotype is distinguished using a unique colour. S1-S15 are the abbreviation for $S1_{Ind}$ -$S15_{Ind}$.

8.3.6. Association between genotype and outcome of infection

After combining all four independent loci, this study identified 41 different genotypes (I1 to I41). Among these genotypes, four were found in multiple isolates, including I6 and I41, which were significantly associated with their respective disease outcomes (**Table no 2**). Three isolates from the asymptomatic group had the I6 genotype, while three isolates from the ALA group had the I41 genotype, with p-values of 0.0183 and 0.0089, respectively. Two isolates in the diarrheal group had the I20 genotype but did not show any significant association with diarrheal development (p value=0.05). Individuals with the I10 genotype were present in both the asymptomatic and ALA groups.

8.3.7. Haplotype networks construction

Haplotype grouping was performed with the NK2 and locus 1-2 markers. There was no apparent effect on isolate grouping when different disease outcome genotypes were included in the haplotype network **(Fig. 8.9 and Fig. 8.10)**. Most of the haplotypes with more than one isolate represented different disease outcome groups. For example, both asymptomatic individuals and amoebiasis patients shared $K2_{Ind}$, $K8_{Ind}$, $K2_{Ind}$ and $K3_{Ind}$ haplotypes of the NK2 locus. E and F haplotypes of locus 1-2 fell in the asymptomatic, diarrhoeal and ALA categories. However, haplotypes $K2_{Ind}$, $K4_{Ind}$, $K5_{Ind}$, $K11_{Ind}$, $K14_{Ind}$, $K16_{Ind}$, $K17_{Ind}$ and $K18_{Ind}$ of the NK2 locus were associated exclusively with a single disease outcome group. Haplotypes F and $K15_{Ind}$ were the most abundant haplotypes in the studied area.

Infection outcome	Sample ID	Sequence pattern				Genotype
		NK2	Locus 1-2	Locus 5-6	SREHP	
Asymptomatic	Kol/A01	$K1_{Ind}$	$L2_{Ind}$	*A5	$S12_{Ind}$	I1
	Kol/A02	$K8_{Ind}$	$L2_{Ind}$	*A5	$S11_{Ind}$	I2
	Kol/A03	$K3_{Ind}$	*B	$Q2_{Ind}$	$S14_{Ind}$	I3
	Kol/A04	$K8_{Ind}$	*B	$Q2_{Ind}$	$S1_{Ind}$	I4
	Kol/A05	$K20_{Ind}$	*F	$Q2_{Ind}$	-	I5
	Kol/A06	$K17_{Ind}$	*F	*A7/$Q7_{Ind}$	$S7_{Ind}$	[b]I6
	Kol/A07	$K17_{Ind}$	*F	*A7 /$Q7_{Ind}$	$S7_{Ind}$	[b]I6
	Kol/A08	$K9_{Ind}$	*F	$Q4_{Ind}$	$S9_{Ind}$	I7
	Kol/A09	$K10_{Ind}$	*E	$Q1_{Ind}$	$S10_{Ind}$	I8
	Kol/A10	$K7_{Ind}$	$L3_{Ind}$	*A7	-	I9
	Kol/A11	$K17_{Ind}$	*F	*A7/ Q7	$S7_{Ind}$	[b]I6
	Kol/A12	$K12_{Ind}$	*F	*A5	$S2_{Ind}$	[#]I10
	Kol/A13	$K3_{Ind}$	$L2_{Ind}$	*A7/$Q6_{Ind}$	$S3_{Ind}$ /$S5_{Ind}$	I11
	Kol/A14	[c]$K5_{Ind}$	*B	*A7/$Q7_{Ind}$	$S14_{Ind}$	I12
	Kol/A15	$K2_{Ind}$	$L3_{Ind}$	*A7/$Q6_{Ind}$	$S13_{Ind}$	I13
Diarrhoea	Kol/D01	$K19_{Ind}$	*B	*A5	$S15_{Ind}$	I14
	Kol/D02	$K2_{Ind}$	$L6_{Ind}$	*A6	$S10_{Ind}$	I15
	Kol/D03	$K4_{Ind}$	$L5_{Ind}$	$Q1_{Ind}$/*A6	$S8_{Ind}$	I16
	Kol/D04	[c]$K14_{Ind}$	*E	*A6/*A7	$S8_{Ind}$	I17
	Kol/D05	[c]$K11_{Ind}$	$L1_{Ind}$	*A5/*A7	$S9_{Ind}$	I18
	Kol/D06	[c]$K18_{Ind}$	$L5_{Ind}$	*A5/*A7	$S8_{Ind}$	I19
	Kol/D07	$K16_{Ind}$	*F	$Q2_{Ind}$/*A7	$S11_{Ind}$	[e]I20
	Kol/D08	$K13_{Ind}$	$L1_{Ind}$	*A5/*A7	$S1_{Ind}$	I21
	Kol/D09	$K15_{Ind}$	*B	*A5/*A7	$S2_{Ind}$	I22
	Kol/D10	$K9_{Ind}$	$L1_{Ind}$	$Q7_{Ind}$	$S12_{Ind}$	I23
	Kol/D11	$K15_{Ind}$	*B	*A5/*A7	$S1_{Ind}$	I24
	Kol/D12	$K15_{Ind}$	*F	$Q4_{Ind}$/$Q5_{Ind}$	$S3_{Ind}$/$S4_{Ind}$	I25
	Kol/D13	$K1_{Ind}$	*B	$Q1_{Ind}$	[c]$S6_{Ind}$	I26
	Kol/D14	$K8_{Ind}$	$L1_{Ind}$	$Q7_{Ind}$	$S7_{Ind}$	I27
	Kol/D15	$K3_{Ind}$	*B	*A5	-	I28
	Kol/D16	$K8_{Ind}$	*B	$Q7_{Ind}$	$S15_{Ind}$	I29
	Kol/D17	$K15_{Ind}$	$L4_{Ind}$	*A5/*A7	$S2_{Ind}$	I30
	Kol/D18	$K6_{Ind}$	$L1_{Ind}$	$Q2_{Ind}$/*A7	$S3_{Ind}$/$S4_{Ind}$	I31
	Kol/D19	$K4_{Ind}$	*B	$Q1_{Ind}$/*A6	-	I32
	Kol/D20	$K9_{Ind}$	$L4_{Ind}$	*A5	$S10_{Ind}$	I33
	Kol/D21	$K20_{Ind}$	$L4_{Ind}$	$Q1_{Ind}$	$S15_{Ind}$	I34
	Kol/D22	$K15_{Ind}$	$L1_{Ind}$	$Q2_{Ind}$/*A7	$S5_{Ind}$	I35
	Kol/D23	$K19_{Ind}$	*F	$Q2_{Ind}$	-	I36
	Kol/D24	$K16_{Ind}$	*F	$Q2_{Ind}$/*A7	$S11_{Ind}$	[e]I20
	Kol/D25	$K8_{Ind}$	$L6_{Ind}$	$Q1_{Ind}$	$S8_{Ind}$	I37
	Kol/D26	$K2_{Ind}$	*F	$Q4_{Ind}$ /$Q5_{Ind}$	$S3_{Ind}$/$S4_{Ind}$	I38
	Kol/D27	$K10_{Ind}$	$L6_{Ind}$	$Q4_{Ind}$ /$Q5_{Ind}$	$S3_{Ind}$/$S5_{Ind}$	I39
Amoebic liver abscess	Kol/ALA01	$K6_{Ind}$	$L3_{Ind}$	Q3 $_{Ind}$	$S9_{Ind}$	I40
	Kol/ALA02	$K7_{Ind}$	$L3_{Ind}$	$Q4_{Ind}$/$Q5_{Ind}$	$S11_{Ind}$	[a]I41
	Kol/ALA03	$K15_{Ind}$	$L2_{Ind}$	*A5	$S4_{Ind}$	I42
	Kol/ALA04	$K6_{Ind}$	$L3_{Ind}$	*A5	$S15_{Ind}$	I43
	Kol/ALA05	$K19_{Ind}$	*E	*A7	-	I44
	Kol/ALA06	$K13_{Ind}$	*E	*A7	$S13_{Ind}$	I45
	Kol/ALA07	$K6_{Ind}$	$L5_{Ind}$	$Q3_{Ind}$	$S15_{Ind}$	I46
	Kol/ALA08	$K7_{Ind}$	$L1_{Ind}$	$Q2_{Ind}$	$S1_{Ind}$	I47
	Kol/ALA09	$K12_{Ind}$	*F	*A5	$S2_{Ind}$	[#]I10
	Kol/ALA10	$K7_{Ind}$	$L3_{Ind}$	$Q4_{Ind}$/$Q5_{Ind}$	$S11_{Ind}$	[a]I41
	Kol/ALA11	$K15_{Ind}$	*E	*A5	$S2_{Ind}$	I48
	Kol/ALA12	$K7_{Ind}$	$L3_{Ind}$	$Q4_{Ind}$/$Q5_{Ind}$	$S11_{Ind}$	[a]I41

* Already reported pattern. [b] Statistically associated with the asymptomatic group, p=0.0183 (fisher's exact test).
[c] Single occurrence observed for these patterns. [#] Observed in both asymptomatic and ALA infection groups.
[a] Statistically associated with ALA, p=0.0089 (fisher's exact test). [e] multiple occurrences in the diarrheal group but not statistically significant.

Table 2 List of identified genotypes (I1 to I41) resulting from the combination of four independent studied loci.

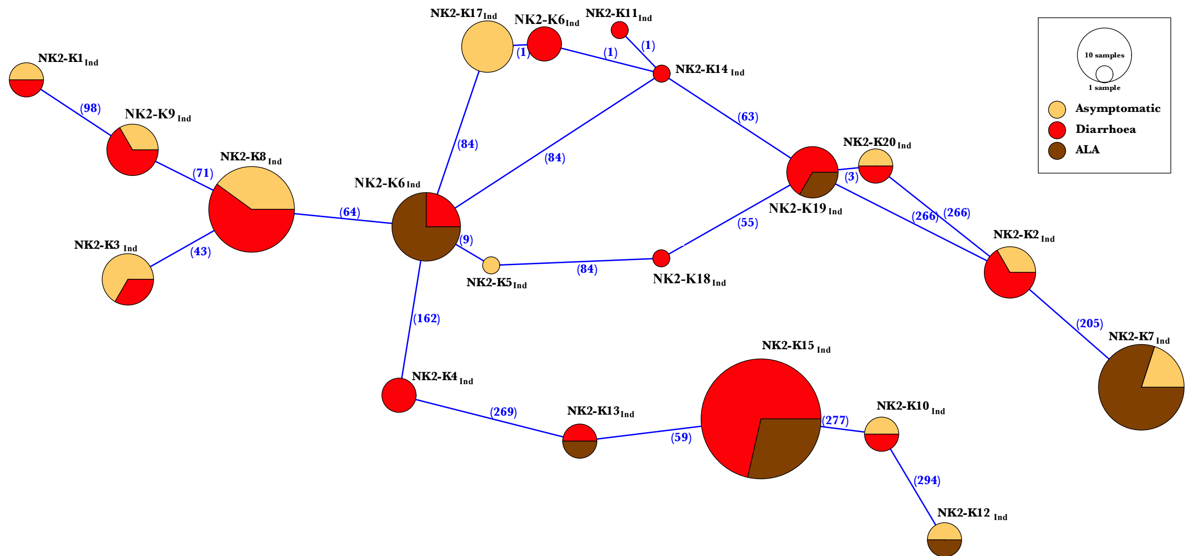


Fig. 8.10 Median-joining network of *E. histolytica* haplotypes obtained from individuals with different disease outcomes groups based on the N-K2 locus. The network illustrates the genetic relationships between the haplotypes, with each circle representing a unique haplotype and the size of the circle indicating its frequency. The colours of the circles correspond to the different disease outcomes. The colours of the circles correspond to the different disease outcome groups.

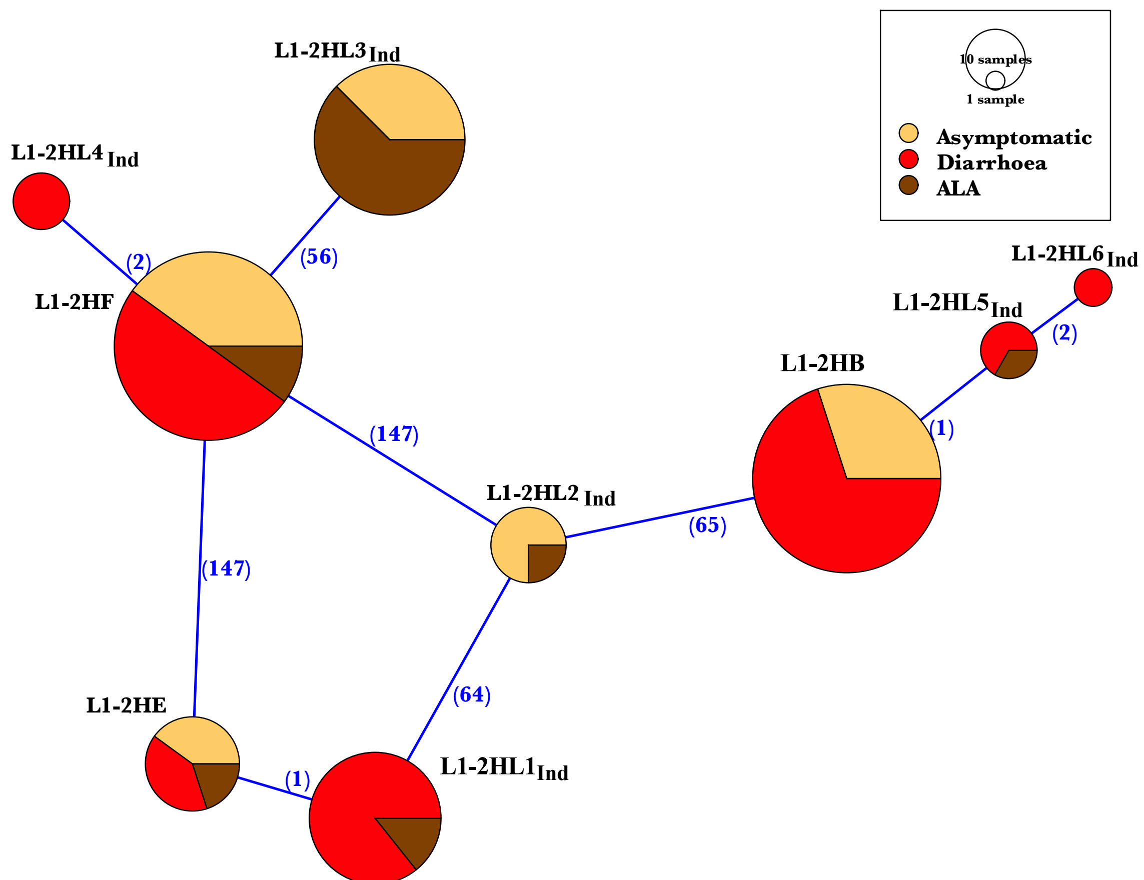


Fig. 8.10 Median-joining network of *E. histolytica* haplotypes obtained from individuals with different disease outcomes groups based on the locus 1-2. The network illustrates the genetic relationships between the haplotypes, with each circle representing a unique haplotype and the size of the circle indicating its frequency. The colours of the circles correspond to the different disease outcomes. The colours of the circles correspond to the different disease outcome groups.

8.4. Discussion

Amoebic infection can be highly risky and is often linked to poor sanitation (Atabati et at. 2020). Other factors that increase the likelihood of infection include handling human or animal waste, exposure to wastewater, engaging in oral-anal sexual practices, and prolonged institutionalization with psychiatric illness or mental retardation (Nishise et al. 2010). Current research on amoebic infection is focused on understanding the genetic variation of different strains of *E. histolytica* and how they cause varying degrees of symptoms (Das et al. 2014). It is essential to gather information about the genomes of infecting strains from areas where the disease is prevalent to better understand how the parasite-genome influences disease outcomes (Zaki et al. 2002). Although various new genotypes of the parasite have been characterized from diverse geographical locations, it is still difficult to anticipate the correlation between the genotype of the parasite and the outcome of the disease. This challenge arises due to the intricate genealogy of the parasite. To address this complexity, we aimed to examine the distribution of repeat patterns (haplotypes) and analyze the association between haplotypes and disease outcomes. The findings of this study may provide insights into the unique lineages of *E. histolytica* present in specific geographic regions and their potential association with disease outcomes. The study highlights the importance of understanding the genealogy of *E. histolytica* to identify potential targets for disease intervention and control. In our study, we conducted genotyping using four genetic markers, including three polymorphic tRNA-linked loci and one coding gene, SREHP. It is widely believed that tRNA genes are susceptible to recombination due to the formation of replication fork barriers by RNA Polymerase III transcription complexes, leading to a pause in replication (Deshpande and Newlon, 1996; Labib and Hodgson, 2007). Additionally, the intergenic regions containing tRNA genes are rich in A+T content, which increases the likelihood of tandem duplication through chance mutations. Notably, the number of short tandem repeats (STRs) in the intergenic tRNA regions has remained relatively stable in *E. histolytica* over several years in a particular strain propagated in a continuous culture. These unique characteristics make these markers highly valuable for assessing the evolutionary divergence of this parasite.

The genome of *E. histolytica* contains a high number of tRNA genes, approximately 4,500 copies, which are arranged in unique tandem repeats forming over 10% of the genome (Tawari et al. 2008). In 2002, Haghighi et al. conducted a study on the correlation between genotypes and clinical presentation in certain gene loci, but they did not find any significant relationship. However, in a later study in 2007, Ali et al. used a different system based on six tRNA gene-

linked STRs and found that the prevalence of parasite genotypes differed significantly among patients with different disease outcomes, indicating a potential role of genotype in disease outcome. Similarly, other studies by Escueta-de Cadiz et al. in 2010, Jaiswal et al. in 2014, and Das et al. in 2014 also found associations between genotypes and clinical phenotypes of amoebiasis using typing of different STRs loci. Although Simonishvili et al. in 2005 found divergent genotypes in the SREHP gene, they did not find any single genotype associated with hepatic disease (Simonishvili et al. 2005). Overall, the correlation between genotypes and pathogenic outcomes in amoebiasis is not yet fully understood. The aim of our study is to gain insights into the genetic patterns and diversity of *E. histolytica* isolates obtained from different disease outcomes using specific genetic markers. To achieve optimal genotyping, we have included the protein-coding gene SREHP along with tRNA-linked 1-2 locus, 5-6 locus, and NK2 locus.

The study has found a large number of unique repeating patterns that are specific to the examined geographic areas. These patterns can be beneficial in conducting global genealogical research on *E. histolytica*. The study samples were analyzed by combining the target loci to identify specific genotypes and then examined to determine if there were any statistically significant associations between the genotypes and disease outcomes. This study revealed that a few genotypes were statistically associated with ALA and the asymptomatic group, but no genotypes were associated with diarrheal outcomes. The observation of the genotype present in both the asymptomatic and ALA groups suggests the possibility of extraintestinal manifestations in asymptomatic carriers of amoebic infection, which supports a similar finding obtained by Das et al. in 2014. Although most of the observed genotypes were single occurrences with no significant correlation to any disease outcomes, multiple occurrences of the same repeat pattern in a particular disease-outcome group could indicate an association of tandem repeat patterns (STR) with disease outcomes. While the sample size in this study was inadequate to estimate the statistical association between genotypes and clinical manifestation, the study recommends genotyping more isolates from different geographical regions to validate these findings. However, it is important to recall that these loci may not have a direct involvement in the virulence of the pathogen, but they could still serve as surrogate markers that are physically linked to other loci that do have a direct effect on the outcome of the infection (Ali et al. 2012).

In addition to identifying exceptional repetitive patterns, we have also discovered several previously unknown repeating units within the studied loci, which is a remarkable finding. The newly obtained sequences with novel repeat units indicate that the isolates are geographically isolated. However, one of the drawbacks we observed in the case of the SREHP gene was that 11.11% of samples were not amplified in SREHP using the primer set. The previous study also recorded a similar observation, indicating its higher genetic polymorphism than the other locus. Therefore, we recommend designing more than one primer set for proper amplification and sequencing to decipher the sequence diversity among isolates and evaluate the association between disease outcome and repeat units in the gene. The observed extensive genetic diversity in the SREHP gene, resulting in its high immunogenicity, implies that the gene may have a vital biological function, such as evading the immune system (Das and Ganguly, 2014).

According to previous studies, there is a closer genetic relationship between amoebic liver abscess isolates and asymptomatic isolates, compared to diarrhoeal isolates of *E. histolytica* (Das and Ganguly, 2014). Our study further supports these findings, as we have identified a genotype that is present in both the asymptomatic and ALA groups. As amoeba trophozoites can cause amoebic liver abscesses by migrating from the intestine to the liver via the bloodstream, individuals with persistent asymptomatic *E. histolytica* infections are at a higher risk of developing this condition in the future (Ali et al. 2007). Therefore, the declining trend of *E. histolytica* infection among diarrheal patients in tropical countries may not necessarily sign of relief, as it could potentially result in an epidemic of ALA. To prevent this outcome, it is crucial to carry out comprehensive investigations of individuals with asymptomatic *E. histolytica* infections (Mukherjee et al. 2009; Mukherjee et.al., 2010; Das et al. 2014). The loci that we analyzed showed extensive genetic polymorphism. While we found a genotype that displayed a statistically significant correlation with ALA, additional studies involving a significant sample size are required to determine the relationship between the other genotypes and the disease outcome of amoebic infection.

This chapter has explored the significance of polymorphic loci in determining the disease outcome of *E. histolytica*. In previous chapters, we have examined the clinical importance of closely related species of *E. histolytica,* namely *E. moshkovskii* and *E. dispar*. In order to address the clinical importance of *E. histolytica, E. moshkovskii*, and *E. dispar*, it is crucial to prioritize the accurate identification and comprehensive characterization of these three species of *Entamoeba*. In the upcoming chapter, we will study the designing and development

strategies specifically tailored to tackle the clinically relevant aspects of these *Entamoeba* species.

8.5. Conclusion

The current study has revealed the genetic makeup of *E. histolytica* isolates in India that are associated with various disease outcomes. The study has identified novel tandem repeats in the analyzed loci, which suggest that *E. histolytica* populations have undergone geographic isolation. Furthermore, our findings support the theory that there is a correlation between parasite genotypes and the outcomes of amoebic infections.

CHAPTER IX

DEVELOPMENT OF HIGHLY SPECIFIC AND SENSITIVE TECHNIQUES FOR DETECTION AND DIFFERENTIATION OF COMMON ENTERIC PARASITES

Development of highly specific and sensitive techniques for detection and differentiation of common enteric parasites

9.1 Background

E. histolytica, G. lamblia, and *Cryptosporidium* spp. are three of the most prevalent enteric parasites responsible for causing diarrhoea worldwide. While microscopy is commonly employed for diagnosing these parasites, its sensitivity and specificity are limited compared to molecular methods. Numerous molecular techniques exist for the simultaneous detection and differentiation of these parasites, each with its own advantages and disadvantages.

For the diagnosis of amoebic infection, microscopy of stool samples has always been considered the "gold standard" technique (Haque et al. 1997; Parija et al. 2014; Tüzemen and Doğan, 2014; Uslu et al. 2016). However, in epidemiological studies of amoebic infection, the species-specific identification and characterization of *Entamoeba* can be challenging due to their identical morphological characteristics, especially in differentiating *E. histolytica, Entamoeba dispar,* and *Entamoeba moshkovskii* (Verweij et al. 2003; Gomes et al. 2014; Calegar et al. 2016; Carrero et al. 2020). New diagnostic techniques are required for the differentiation of *Entamoeba* species (Roshdy et al. 2017).

Recently, the Polymerase Chain Reaction (PCR) technique has been accepted by the World Health Organization (WHO) as an alternative diagnostic tool for the detection of *E. histolytica* (Uslu et al. 2016; Emisiko et al. 2020; Shirley et al. 2018). Nowadays, the importance of other species like *E. dispar* and *E. moshkovskii* is increasing in studies of infection by the pathogenic species *E. histolytica*, as both of them have been reported in human patients over the years (Soares et al. 2019).

It has been reported that approximately 10% of the world's population is infected with *Entamoeba* species, of which the pathogenic *E. histolytica* comprises only 10%, while the remaining 90% persist as the commensal species *E. dispar* (Ngui et al. 2012). Moreover, intestinal colonization of *E. moshkovskii* in humans has been reported in different countries, such as the United States, Australia, Indonesia, Colombia, Malaysia, Italy, Iran, Turkey, Bangladesh, India, Kenya, Tunisia, Tanzania, and Brazil, with gastrointestinal clinical manifestations in some settings (Parija and Khairnar, 2005; Fotedar et al. 2007; Khairnar and

Parija, 2007; Ayed et al. 2008; Beck et al. 2008; Delialioglu et al. 2008; Anuar et al. 2012; Ngui et al. 2012; Shimokawa et al. 2012; Fonseca et al. 2016; Al-Areeqi et al. 2017; Kyany'a et al. 2019). Therefore, the prevalence of *E. histolytica* infection is often overestimated due to its epidemiological overlap with other morphologically identical species, particularly the commensal *E. dispar* and the amphizoic *E. moshkovskii* (Soares et al. 2019).

In diagnosis of amoebiasis, ELISA is more sensitive than microscopic detection techniques but less sensitive and specific than DNA-based assay (Randall et al. 1984; Haque et al. 1998; Delialioglu et al. 2008; Lau et al. 2013). Overall DNA amplification assays (PCR) have been proven to be highly sensitive and specific among other molecular methods (Haque et al. 1998). A wide variety of PCR techniques exist such as nested, multiplex, and real-time PCR, and each has its own advantages and limitations (Haque et al. 1998; Abe et al. 2002; Blessmann et al. 2002). Detection and differentiation of *E. histolytica / E. dispar / E. moshkovskii* by conventional/nested PCR for each species separately is highly sensitive but is a time-consuming procedure and also not at all an economic approach (Souza et al. 2007). While nested PCR assay is extremely sensitive, it is also highly susceptible to contamination (Green and Sambrook, 2019). Moreover, nested PCR is a very time-consuming and costlier procedure (Wang et al. 2014).

In this chapter, we will discuss into the design and development of three DNA-based assays used to detect and differentiate clinically significant enteric parasite species. The designed three assay methods are:

A. Development of a simple PCR-RFLP technique for detection and differentiation of *E. histolytica, E. dispar* and *E. moshkovskii*

B. Development of single round multiplex PCR assay for differentiation of morphologically identical *Entamoeba* species.

C. Development of a TaqMan probe-based multiplex qPCR assay for simultaneous detection of enteric parasites viz. *Giardia spp, Entamoeba spp* (*E. histolytica, E. dispar and E. moshkovskii) and Cryptosporidium* spp.

9.2 Ethical statement

The Institutional Human Ethics Committee of ICMR-NICED approved the protocols for the development of diagnostic methods. Prior to participation, individuals provided informed consent, and their samples were gathered specifically for the purpose of validating the

developed diagnostic methods. Anonymously coded samples were employed to maintain confidentiality and protect the identity of the study participants.

9.3 Development of a simple PCR-RFLP technique for detection and differentiation of *E. histolytica, E. dispar* and *E. moshkovskii*

Multiplex PCR assay is a useful tool for detection and differentiation of different *Entamoeba* species (Evangelopoulos et al. 2000; Abe et al. 2002; Khairnar and Parij, 2007), but standardization of multiplex PCR is very tricky and non-specific amplifications are also a major drawback of this technique (Fotedar et al. 2007). Multiplex PCR assay kit is not commercially available and test centres in endemic areas must standardize or develop their own protocols for the differentiation of the *E. histolytica / E. dispar / E. moshkovskii* complex by using this technique (Fontecha et al. 2015).

Real-time PCR based identification and differentiation of *Entamoeba* spp. is largely being used for both clinical and research purposes with the advantages of increased sensitivity and specificity but this technique is laborious, time-consuming, expensive and requires expertise (Roy et al. 2005; Fotedar et al. 2007). In clinical situations detection and differentiation of each species using separate conventional PCR / Real-time PCR reactions is practically challenging. Therefore, a simpler and better molecular tool is required for the diagnosis of amoebiasis as well as patient care management to avoid unnecessary pharmacological treatment. For understanding the epidemiology of *Entamoeba* spp a highly sensitive and specific technique is also needed (Gomes et al. 2014). In the present study, we have designed, developed and evaluated a simple PCR-RFLP method for detection and differentiation of small quantities of *E. histolytica/ E. dispar* / *E. moshkovskii* DNA from human fecal samples. Considering PCR a better tool, this method will be able to overcome many of the limitations posed by conventional techniques and will be more economical and practical for accurate diagnosis of different species of *Entamoeba* in the epidemiological study of amebiasis outbreaks.

9.3.1 Methodology

9.3.1.1 Molecular identification of *E. histolytica, E. dispar* and *E. moshkovskii* using published primer sets

A total of 382 fresh faecal specimens were collected and tested to evaluate the efficacy of the PCR-RFLP technique as a routine diagnosis of *E. histolytica* infection. The faecal samples were collected from diarrheal patients aged ≥ 5 years to ≤ 60 years admitted in Beliaghata ID hospital, Kolkata by trained medical professionals in the presence of attending physicians on the first day of hospitalization and prior to administration of antibiotics. This study was conducted from March 2019 to October 2019. The protocol of this study was approved by the Institutional Human Ethics Committee of ICMR-National Institute of Cholera and Enteric Diseases, Kolkata. Informed consent for participation was obtained from every patient. In the case of children, consent was obtained from their parents.

Fresh unpreserved samples were immediately sent to laboratories for examination. DNA was extracted from the stool samples using STOOL DNA Minikit (QIAGEN, USA) according to the manufacturer's instructions, and the DNA was kept in a -20°C freezer until further use.

For detection of *E. histolytica,* a set of previously described primer pairs were employed, EH1 5'-GTACAAAATGGCCAATTCATTCAATG-3' as forward primer and EH2 5'-ACTACCAACTGATTGATAGATCAG-3' as reverse primer (Mukherjee et al. 2009). The EH1 and EH2 oligonucleotides generated 135 bp products in presence of *E. histolytica* DNA. *E. dispar* and *E. moshkovskii* were also identified by previously described nested PCR method. For *E. moshkovskii* identification the primers used were Em_F1 5'CTCTTCACGGGGAGTGCG-3', Em_R1 5'TCGTTAGTTTCATTACCT-3', Em_nF2 5'GAATAAGGATGGTATGAC-3' and Em_nR2 5'AAGTGGAGTTAACCACCT-3' (Ali et al. 2003). Nested PCR for *E. moshkovskii* produced 258 bp amplicons. For *E. dispar* the primers were E-1 5' AAGCATTGTTTCTAGATCTGAG-3' (forward primer), E-2 5' AAGAGGTCTAACCGAAATTAG-3' (reverse primer), nED-1 5' TCTAATTTCGATTAGAACTCT (forward primer) 3', nED-2 5' TCCCTACCTATTAGACATAGC 3' (reverse primer) (Bahrami et al. 2019). Nested PCR using nED-1 and nED-2 oligonucleotides generated amplicons of 174 bp in the presence of *E. dispar* DNA. Conditions for the abovementioned PCR amplifications were mentioned elsewhere (Ali et al. 2003; Mukherjee et al. 2009; Bahrami et al. 2019).

9.3.1.2 Primer design

Partial *18S rRNA* gene sequences were selected for primer designing in this study. *18S rDNA* sequences of *Entamoeba* spp. were retrieved from the NCBI database and aligned using the ClustalW program (https://www.genome.jp/tools-bin/clustalw). We analysed the following sequences: *E. histolytica*, GenBank accession no. X56991 (1947 bp); *E. dispar*, GenBank

accession no. AB282661.1 (2426 bp); *E. moshkovskii*, GenBank accession no. AF149906 (1944 bp); *Entamoeba coli* Grassi, 1879 , GenBank accession no. AF149914 (2101 bp); *Entamoeba chattoni* Levine, 1961, GenBank accession no. AF149912 (1963 bp); *E. polecki* Prowazek, 1912, GenBank accession no. AF149913 (1858 bp); *Entamoeba invadens* Rodhaim 1934, GenBank accession no. AF149905 (1965 bp). Highly conserved regions for *E. histolytica/ E. dispar/ E. moshkovskii* complex were recognised and assessed for primer designing. Two regions were selected for a pair primer which was able to amplify *E. histolytica/ E. dispar/ E. moshkovskii* complex DNA. The primer sequences were as follows: forward primer, EHEDEM_F (5′-AAAGACCAAGTAGGATGAAACTGC-3′) and reverse primer, EHEDEM_R (5′-TTCCTTCTACTGTTCGGTCTTG-3′). Length and melting of the primers were verified. Basic local alignment search tool in the NCBI base was also used to make sure of unique specificity. The designed primer set *al*so may amplify *Entamoeba nuttalli* Catellani, 1908 (it has been called '*E. histolytica* like variant' and *E. histolytica* Non-Human Primates/ NHPs variant by some) DNA as per sequences available on AmoebaDB, but this species was not included in our analysis. Note that most DNA-based diagnostic techniques are unable to differentiate *E. nuttalli* from *E. histolytica*, if not combined with sequencing, and neither can some commercial antigen-based assay kit and monoclonal antibodies (Stensvold et. al. 2011; Elsheikha et. al. 2018). Moreover, clinical importance of *E. nuttalli* in human amoebiasis is still not well-defined (Tachibana et. al. 2009; Levecke et. al. 2015; Elsheikha et. al. 2018).

9. 3.1.3 Genomic DNA isolation

Genomic DNA of *E. histolytica* (HM1:IMSS clone 6) and *E. moshkovskii* (Laredo strain) were isolated directly from the routinely maintained axenic culture in our laboratory. The *E. moshkovskii* strain was a gift from Dr Seiki Kobayashi, Keio University, School of Medicine. DNA isolation was carried out using GENOMIC DNA Minikit (QIAGEN, USA) as per the manufacturer's protocol. The genomic DNA of *E. dispar* was also provided by Dr Seiki Kobayashi, School of Medicine, Keio University and the DNA was isolated as previously described. Genomic DNA used in this study was isolated from trophozoites under log phase of growth.

9. 3.1.4 PCR amplification

E. histolytica/ E. dispar/ E. moshkovskii complex specific amplifications were performed using Applied Biosystem 9700 thermal cycler in a total of 50 µl reaction mixture for 35 cycles. To ensure desired amplification of the target loci, this *E. histolytica/ E. dispar/ E. moshkovskii*

complex-specific PCR was adjusted. 72°C is the optimum extension temperature for the highest intensity of PCR products. The optimal primer concentration for both of the primers was 0.2 μM. After running a gradient PCR, 55°C was observed as an optimal annealing temperature. Overall the PCR conditions involved at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 56°C for 25 s, and extension at 72°C for 40 s. The amplification reactions mixture contained 1.25U Takara Ex Taq DNA polymerase and 2.0 μl of DNA. PCR was performed in a final concentration of 1X Ex Taq buffer and 0.2 mM Ex Taq dNTP. PCR products were separated by electrophoresis in 1% agarose gels and visualized using a UV transilluminator after 0.5 μM/ml of ethidium bromide staining. The expected amplicon size of the PCR was 1191 bp/1193 bp in the presence of *E. histolytica/ E. dispar/ E. moshkovskii* complex.

9. 3.1.5 Specificity assay of the primers

To assess the non-target amplification of *E. histolytica/ E. dispar/ E. moshkovskii* complex specific primer set, DNA isolated from stool samples positive for *E. coli, E. invadens, E. polecki, E. chattoni, Cryptosporidium parvum*, *Giardia lamblia*, and mixed bacterial infections were used as template in the optimized reaction system. Reference DNA of *E. histolytica, E. dispar, and E. moshkovskii* were also used during the specificity assay.

9. 3.1.6 Sensitivity assay of primers

To evaluate the sensitivity of the primer set, reference DNA templates of *E. histolytica, E. dispar* and *E. moshkovskii* were serially diluted to 10ng/ μL, 5 ng/μL, 2.5 ng/μL, 1.25 ng/μL, 0.625 ng/μL, 0.3125 ng/μL, 0.1563 ng/μL, 0.07813 ng/μL, 0.0391 ng/μL, 0.0195 ng/μL, 0.0098 ng/μL, 0.0049 ng/μL, 0.0025 ng/μL, 0.0013 ng/μL by two-fold gradient with nuclease free water. Then PCR amplifications were carried out as per the aforesaid protocol. The detection limit of the PCR-RFLP was evaluated using the sensitivity assay.

9. 3.1.7 Restriction Fragment Length Polymorphism (RFLP) Analysis

All the stool DNA extracts were amplified again following the abovementioned PCR method to perform our PCR-RFLP method. To differentiate the three species of *Entamoeba*, the *restriction map* was generated *with* GenScript Restriction Enzyme Map Analysis Tools based on the expected nucleotide sequences of *E. histolytica, E. dispar* and *E. moshkovskii.* For species differentiation, restriction enzyme Tat1 was selected to digest the PCR products.

Tat1 restriction enzyme recognizes and cleaves DNA at 5’-W↓GTACW-3’/ 3’-WCATG↑W-5’. After the digestion of PCR products, Tat1 showed three different RFLP patterns for three species. Based on the reference 18S rDNA sequences, the expected RFLP pattern for *E. histolytica* was 125 bp, 850 bp and 216 bp fragments; for *E. dispar*, 66 bp/59 bp (co-migrating bands due to a small difference in length), 852 bp and 216 bp fragments, whereas for *E. moshkovskii*, 66 bp/59 bp (co-migrating bands) and 1066 bp fragments (**Fig. 9.1**). Restriction digestion was carried out using 1.25U of Tat1 (Thermofisher Scientific) restriction enzyme. Briefly, 12 µl aliquot of the amplified PCR product and 0.25 µl of the Tat1 restriction enzyme was prepared by mixing 2 µl of 10x Tango buffer (Thermofisher Scientific), and 5.75 µl nuclease-free water was added to make the total volume of 20 µl. Tat1 reactions were incubated at 65°C for 10 mins following the instructions of the manufacturer. The digested PCR products were separated by electrophoresis through 1.5% agarose gel and visualized by 0.5 µM/ml of ethidium bromide staining under a UV transilluminator.

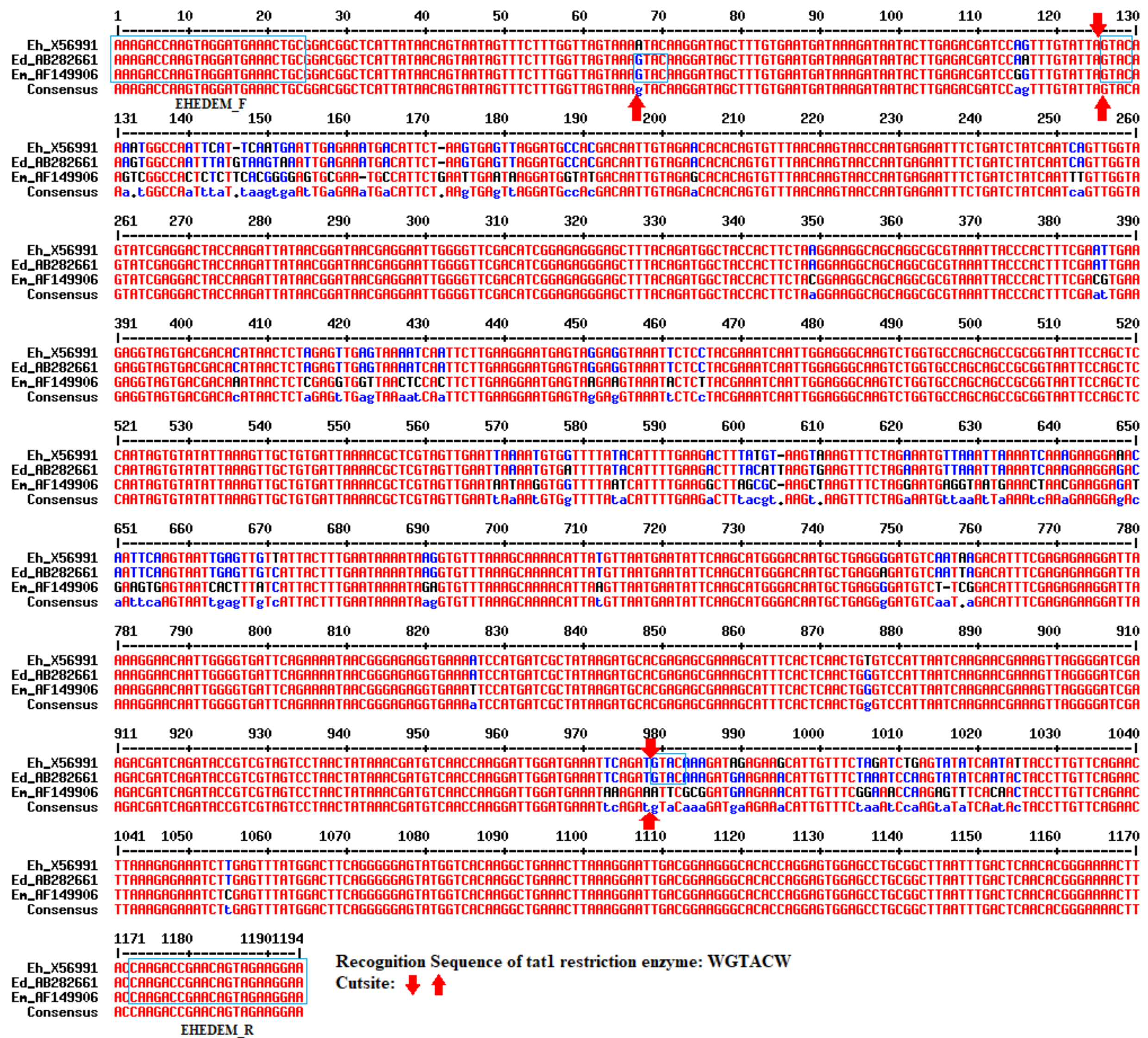


Fig. 9.1 Multiple alignment showing primer flanking regions for EHEDEM_F and EHEDEM_R primer set in targeted 18S rRNA locus of three *Entamoeba* reference strains viz. *E. histolytica* (GenBank: X56991), *E. moshkovskii* (GenBank: AF149906) and *E. dispar* (GenBank: AB282661) using MultAlin online tool. Arrows indicate the location of Tat1 restriction sites.

9. 3.1.8 Validation of the assay by sequencing

A part of the positive PCR product was purified with High Pure PCR Product Purification Kit (Roche, Switzerland) as per the manufacturer's instructions. The purified PCR products were then sequenced bidirectionally with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a DNA analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were edited manually after using the clustalW multiple sequence alignment program of GenomeNet Bioinformatics tools. For similarity search with *Entamoeba* spp. all the obtained sequences were analysed in Genbank database using the BLAST program on the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results obtained in the PCR-RFLP method were compared with the results of sequencing and evaluated the accuracy of the PCR-RFLP method. These results indicate that the designed strategy has a higher specificity.

9.3.1.9 Validation of the assay comparing data with the previously described primer set

The result obtained in the PCR-RFLP method was also compared with the data obtained from previously published primer set (Ali et al. 2003; Mukherjee et al. 2009; Bahrami et al. 2019) of each species for estimation of the accuracy of the PCR-RFLP method.

9. 3.1.10 Statistical analysis

Confidence intervals were computed by the modified Wald method using GraphPad Prism, USA online resources.

9. 3.2 Results

9.3.2.1 Occurrence of *E. histolytica, E. dispar* and *E. moshkovskii*

Out of 382 clinical fecal samples, 1.05% (n= 4/382, 95% CI 0.0031 - 0.0276) were identified as *E. histolytica*; 0.52% (n= 2/382, 95% CI 0.0002 - 0.0202) were identified as *E. dispar* and 2.62% (n= 10/382, 95% CI 0.0136 - 0.0482) were identified as *E. moshkovskii*. Therefore, the overall infection rate of *E. histolytica/ E. dispar/ E. moshkovskii* complex was 4.19% (n=16/382, 95% CI 0.0254 - 0.0674). All of species were identified by PCR techniques using the aforementioned primer set (**Fig. 9.2**, **Fig. 9.3** and **Fig. 9.4**).

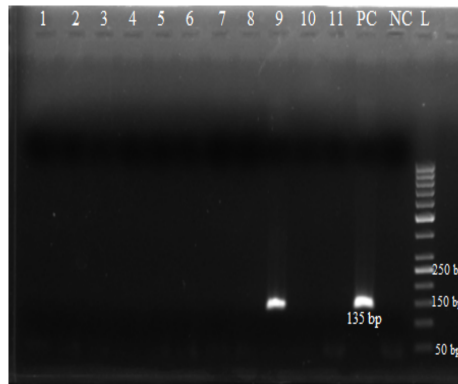


Fig. 9.2 Detection of *E. histolytica* based on 18SrRNA locus using previously described PCR assay. PCR product size: 135 bp. Lanes 1-11 contains PCR product of diarrhoeal stool samples DNA collected from ID Hospital, Kolkata. PC: Positive control contains *E. histolytica* DNA, NC: Negative control, Lane 9 is positive for *E. histolytica.*

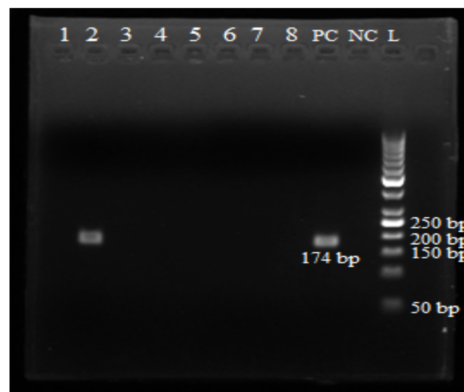


Fig. 9.3 Detection of *E. dispar* based on 18SrRNA locus using previously described nested PCR assay. Nested PCR product size: 174 bp. Lanes 1-8 contains PCR product of diarrhoeal stool samples DNA collected from ID Hospital, Kolkata. PC: Positive control contains *E. dispar* DNA, NC: Negative control, Lane 2 is positive for *E. dispar.*

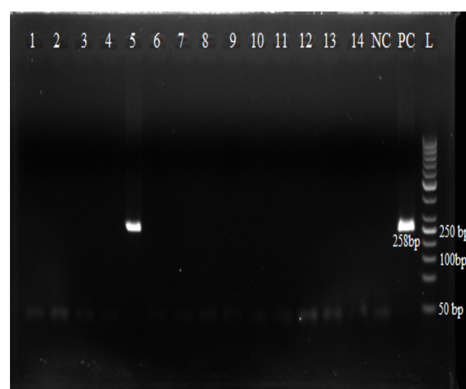


Fig. 9.4 Detection of *E. moshkovskii* based on 18SrRNA locus using previously described nested PCR assay. Nested PCR product size: 258 bp. Lanes 1-15 contain PCR product of diarrhoeal stool samples DNA collected from ID Hospital, Kolkata. PC: Positive control contains *E. dispar* DNA, NC: Negative control, Lane 5 is positive for *E. dispar.*

9.3.2.2 Methodological efficacy

E. histolytica/ E. dispar/ E. moshkovskii complex-specific primer set successfully produced 1191 bp PCR products in the presence of the reference strains *E. histolytica* and *E. moshkovskii* (**Fig. 9.5**). The primer set also produced an 1193 bp amplicon in presence of reference strain *E. dispar* (**Fig. 9.5**). We did not observe any non-specific amplification in case of stool DNA extracted from positive samples for *E. coli, E. invadens, E. polecki, E. chattoni, Cryptosporidium parvum*, *Giardia lamblia*, and mixed bacterial infections (**Fig. 9.6**). Sensitivity assay of the primer pair showed that it was able to amplify as little as 2.6 pg genomic DNA of *E. histolytica, E. dispar* and *E. moshkovskii* with high band intensity (>20 ng/µl) (**Fig. 9.7**). Therefore, the designed primer set was sensitive enough to properly identify *E. histolytica/ E. dispar/ E. moshkovskii* complex. Furthermore, restriction digestions of PCR products with Tat1 produced expected DNA fragment patterns as predicted by *restriction map*ping. Digestion of PCR products of *E. histolytica* genomic DNA generated fragments of 125 bp, 850 bp and 216 bp; that of *E. dispar* genomic DNA generated 66 bp/59 bp (co-migrating bands), 852 bp and 216 bp fragments; and *E. moshkovskii* genomic DNA generated 66 bp/59 bp (co-migrating bands) and 1066 bp fragments (**Fig. 9.8**). This result indicated that the restriction pattern of Tat1 could easily differentiate *E. histolytica, E. dispar* and *E. moshkovskii* successfully.

Fig. 9.5 Amplification of 18SrRNA locus of different *Entamoeba* species using EHEDEM_F and EHEDEM_R primer set; 1: *E. histolytica* 1191 bp, 2. *E. dispar* 1193 bp, 3: *E. moshkovskii* 1191 bp, NC: Negative control; L: 1 kb Ladder.

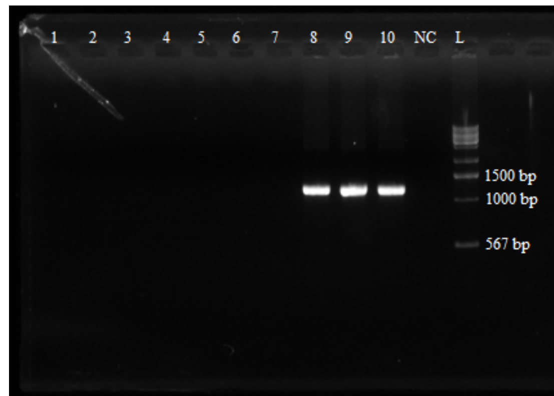


Fig. 9.6 Specificity checking of the EHEDEM_F and EHEDEM_R primer set with other well known enteric pathogens; 1: *E. coli* DNA, 2: *E. invadens* DNA, 3: *E. polecki* DNA, 4: *E. chattoni* DNA, 5:*Cryptosporidium parvum* DNA, 6: *Giardia lamblia* DNA, 7: DNA from mixed bacterial infections , 8: *E. histolytica* DNA, 9. *E. dispar* DNA, 10: *E. moshkovskii* DNA, NC: Negative control; L: 1 kb Ladder.

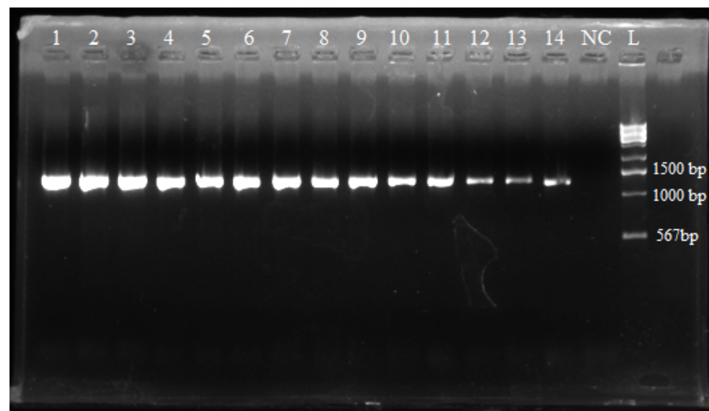


Fig. 9.7 Sensitivity of *E. histolytica/ E. dispar/ E. moshkovskii* complex specific PCR assay with twofold serial dilutions of *E. histolytica* DNA. Lane 1-14> PCR products of reference strain DNA; 10ng, 5 ng, 2.5 ng, 1.25 ng, 0.625 ng, 0.3125 ng, 0.1563 ng, 0.07813 ng, 0.0390 ng, 0.01953 ng, 0.0098 ng, 0.0049 ng, 0.0023 ng, 0.0012 ng. NC> Negative control, L> 1 kb ladder.

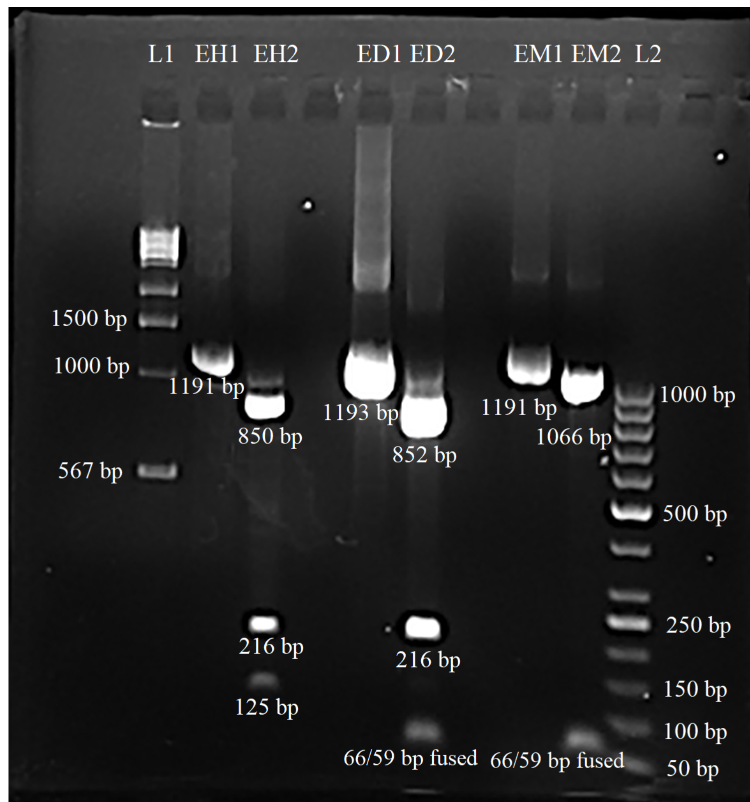
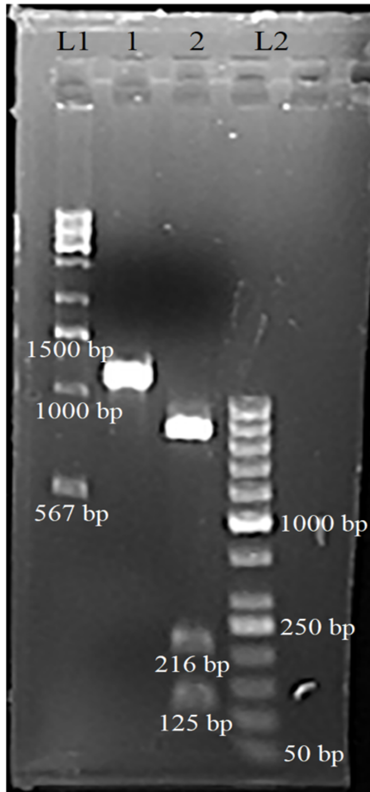
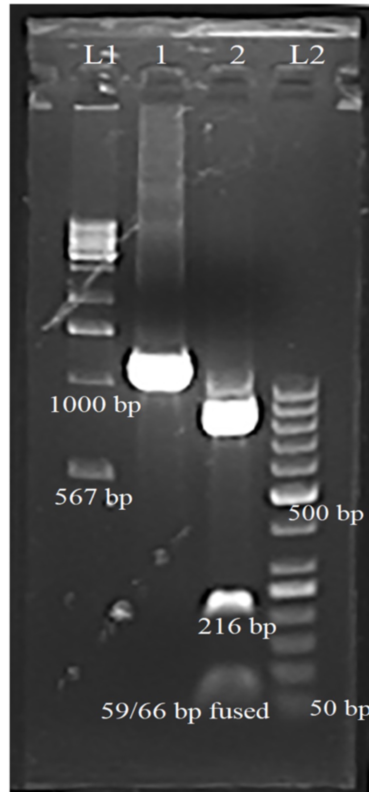


Fig. 9.8 The result of PCR-RFLP after digestion with Tat1 restriction enzyme on the selected strains of *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii* are: Lanes EH1>Amplified PCR product of *E. histolytica* reference strain based on 18SrRNA locus, EH2> Restriction patterns (125 bp, 850 bp and 216 bp) of the 18S rRNA gene fragment amplified from the total DNA of *E. histolytica* culture; Ed1>Amplified PCR product of cultured *E. dispar* strain based on 18SrRNA locus, Ed2> Restriction patterns (66 bp/ 59 bp fused band, 852 bp and 216 bp) of the 18S rRNA gene fragment amplified from the total DNA of *E. dispar* culture; EM1> Amplified PCR product of *E. moshkovskii* reference strain based on 18SrRNA locus, EM2> Restriction patterns (66 bp/ 59 bp fused band and 1066 bp) of the 18S rRNA gene fragment amplified from the total DNA of *E. moshkovskii* culture; L1>1 kb ladder, L2> 50 bp ladder.

9.3.2.3 Detection of *Entamoeba* complex using PCR-RFLP method in clinical Samples

An 18S rDNA fragment was successfully amplified in 16 out of 382 diarrhoeal stool specimens (4.19%) by using *E. histolytica/ E. dispar/ E. moshkovskii* complex-specific primer set. *Entamoeba* species were identified by digestion of the PCR products with Tat1 restriction enzyme using the aforesaid protocol. Tat1 restriction enzyme digestion produced an expected electrophoretic pattern depending upon the presence of *Entamoeba* species. After PCR-RFLP 1.05% of samples generated *E. histolytica-specific* DNA fragment patterns, 0.52% of samples generated *E. dispar*-specific DNA fragment patterns and 2.62% of samples generated *E. moshkovskii*-specific DNA fragment patterns (**Fig. 9.9**, **Fig. 9.10 and Fig. 9.11**). Therefore, the result obtained by the PCR-RFLP method was exactly similar to the previously described molecular method viz. PCR/nested PCR assay. It is also noteworthy that we did not observe any remarkable non-specific DNA amplification during PCR (using EHEDEM_F and EHEDEM_R primer set) of the stool samples. Furthermore, the digestion of positive PCR products with the Tat1 enzyme did not produce any undesirable DNA fragment patterns.





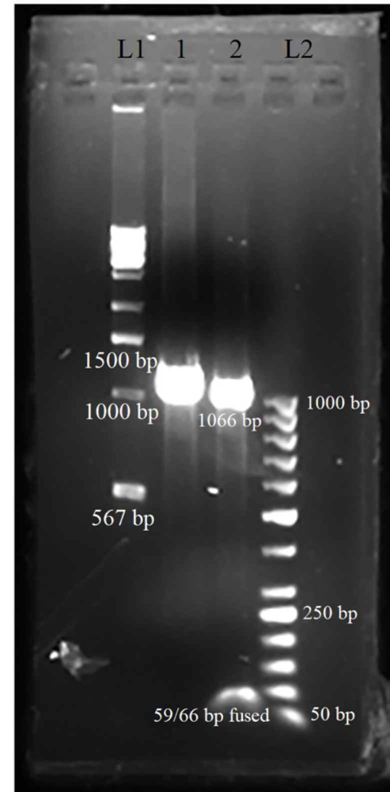


Fig. 9.9 **Fig. 9.10** **Fig. 9.11**

Fig. 9.9 The result of PCR-RFLP after digestion with Tat1 restriction enzyme on clinical sample: Lane 1> Amplified PCR product of *E. histolytica* clinical sample based on 18SrRNA locus; Lane 2> Restriction patterns of the 18S rRNA gene fragment amplified from the total DNA of *E. histolytica* positive stool sample. L1>1 kb ladder, L2> 50 bp ladder.

Fig. 9.10 The result of PCR-RFLP after digestion with Tat1 restriction enzyme on clinical sample: Lane 1> Amplified PCR product of *E. dispar* clinical sample based on 18SrRNA locus; Lane 2> Restriction patterns of the 18S rRNA gene fragment amplified from the total DNA of *E. dispar* positive stool sample. L1>1 kb ladder, L2> 50 bp ladder.

Fig. 9.11 The result of PCR-RFLP after digestion with Tat1 restriction enzyme on clinical sample: Lane 1> Amplified PCR product of *E. moshkovskii* clinical sample based on 18SrRNA locus; Lane 2> Restriction patterns of the 18S rRNA gene fragment amplified from the total DNA of *E. moshkovskii* positive stool sample. L1>1 kb ladder, L2> 50 bp ladder.

9.3.2.4 Clinical evaluation of PCR-RFLP using DNA sequencing

All the 16 PCR-positive samples for *E. histolytica/ E. dispar/ E. moshkovskii* complex were validated with Sanger sequencing. The BLAST sequence analysis tool (NCBI) revealed three different *Entamoeba* species: *E. histolytica* (n=4), *E. dispar* (n=2) and *E. moshkovskii* (n=4). Therefore, sequencing results were found to be consistent with the PCR-RFLP method. Representative sequences obtained in this study were deposited in GenBank under the accession numbers OM780323 - OM780326, OM791700 - OM791701 and OM791622 - OM791631.

9.3.2.5 Detection limits

The sensitivity assay showed that the differentiation limits of each *Entamoeba* species using the PCR-RFLP method were as low as 5 pg genomic DNA in the PCR reaction mixture. This concentration of genomic DNA was able to produce an easily visible RFLP pattern after restriction digestion. Although a very faint band was seen with as low as 2.6 pg genomic DNA using the primer set, but could not produce a visible RFLP pattern.

9.3.2.6 Sequence analysis of clinical samples

To confirm the efficacy of the developed strategy, amplified products of 16 positive samples were purified and sequenced. After sequencing, we observed that 87.50% (n/N=14/16, 95% CI 0.6272 - 0.9776) samples showed 100% similarity to corresponding reference strain sequences in the GenBank database. Four *E. moshkovskii* isolates were exceptions having three nucleotide substitutions (312A/T, 677A/C, 690T/C). Substitution 312A/T was observed in three isolates (EM/KOL_003, EM/KOL_004, and EM/KOL_007), whereas substitution 677A/C and 690T/C were observed in one isolate (EM/KOL_008). The substitutions were associated with transversion (Pyrimidine<=>purine) mutation. But the change of the nucleotide sequences due to substitutions did not affect the restriction digestion of the Tat1 enzyme.

9.3.4 Discussion

In this study, we successfully developed a new method for the detection and differentiation of E. *histolytica, E. dispar* and *E. moshkovskii*. Our PCR-RFLP assay is simple, concise, easily accessible, labour saving and economical which might be a useful ancillary approach in the clinical diagnosis of amoebiasis. This technique was successfully applied for species differentiation in *E. histolytica/ E. dispar/ E. moshkovskii* complex of 382 diarrhoeal patients

from Kolkata, India with substantially reduced time when compared to single multiple PCR reactions.

For diagnosis of intestinal amoebiasis, microscopic examination has always been the most common strategy in many countries (Uslu et al. 2016). The microscopic study cannot differentiate between morphologically indistinguishable species like *E. histolytica*, *E. dispar*. and *E. moshkovskii* (Gomes et al. 2014; Calegar et al. 2016; Roshdy et al. 2017; Emisiko et al. 2020). Detection of *Entamoeba* complex must be performed within the first hour of collection of stool samples for examining the motile trophozoites (Al-Dalabeeh et al. 2020). Epithelial cells, macrophages and many other structures such as undigested food particles can be confused for cysts or trophozoites of *Entamoeba* species (Al-Dalabeeh et al. 2020). Three successive stool specimens are recommended for an accurate diagnosis of intestinal amoebiasis. Additionally, microscopic detection of *E. histolytica* mostly depends on the skills and experiences of the microscopist (Verweij et al. 2003; Al-Dalabeeh et al. 2020). Therefore, microscopic identification of cysts and trophozoites of *E. histolytica* is likely to generate false positive reports which may encourage unnecessary treatment with amoebicidal drugs (Al-Dalabeeh et al. 2020). Due to these limitations, a number of molecular diagnostic techniques like conventional PCR, RT-PCR, and ELISA have been evaluated for appropriate diagnosis of amoebiasis (Randall et al. 1984; Haque et al. 1998; Abe et al. 2002; Blessmann et al. 2002; Hamzah et al. 2006; Delialioglu et al. 2008; Lau et al. 2013). But most of techniques have several limitations as already mentioned (Haque et al. 1998; Abe et al. 2002; Blessmann et al. 2002; Hamzah et al. 2006). Therefore, a simple, highly sensitive and specific screening test is needed for rapid and accurate diagnosis of amoebic infection. In this respect, we have successfully designed, developed and evaluated the new simple PCR-RFLP method for the detection and differentiation of the three *Entamoeba* species from stool specimens. This method will increase efficiency and versatility for the diagnosis of amoebic infection.

The present study illustrates a new PCR-RFLP approach for species-specific detection and differentiation of three morphologically identical species of *Entamoeba* viz. *E. histolytica, E. moshkovskii* and *E. dispar* (**Fig. 9.12**). In this method, we have applied two molecular techniques, a conventional PCR for the detection of *E. histolytica/ E. dispar/ E. moshkovskii* complex and an RFLP for the differentiation of the three species.

All the 382 clinical samples were initially screened using the conventional/nested PCR method for the three species, we further evaluated the samples using the described PCR-RFLP strategy. The other parasites except *Entamoeba* complex were excluded during PCR-based preliminary

screening. Additionally, it was noted that the primer pair was highly sensitive and amplified *Entamoeba* complex DNA with less than 1 ng. Overall, the result shows that the conventional/ nested PCR method provided exactly similar results compared to our PCR-RFLP method for the identification of each species separately without any significant difference observable. Moreover, sequencing results completely corresponded to the outcomes of the PCR-RFLP method which indicates an equal specificity of the developed strategy with PCR detection techniques. Since the 18S rRNA gene is a highly conserved component and shows minimal nucleotide substitution rates, the differentiation of species using the 18S rRNA sequence-based PCR-RFLP method will be markedly specific and a practical approach (Fontecha et al. 2015). Additionally, almost 200 copy number of the rDNA gene per haploid genome have been estimated in exponentially growing *E. histolytica* HM1: IMSS cells and the rDNA copies do not show any heterogeneity (Bhattacharya and Bhattacharya, 2013). Therefore, due to the abundance of this DNA locus in the cell, the 18S rRNA sequence-based PCR-RFLP strategy will be sensitive enough for diagnostics. Furthermore, PCR-RFLP has additional advantages over single multiple PCR reactions for species-specific identification, such as it is able to detect and differentiate the three species in a single enzyme reaction assay and is suitable for rapid diagnosis of a large number of samples. A limitation of our PCR-RFLP strategy is that it demands amplified PCR products with high band intensity (>50ng/µl) for easily visible RFLP patterns in agarose gel. This method can be implemented in the laboratory easily and does not require advanced expensive equipment. It is best suited for the investigation of a large number of samples for diagnosis of amoebic infection as it can easily and accurately differentiate all three *Entamoeba* species using a single marker. Overall this method is a highly reproducible strategy for profiling and identification of parasite populations. This analysis indicates that this strategy is reliable for identifying *E. histolytica, E. moshkovskii* and *E. dispar* from faecal specimens. However, further analysis needs to be carried out, such as studies in wider geographical areas with adequate samples from different host species and genotyping samples for an entire year to reveal the applicability of the technique in diagnostics in the near future.

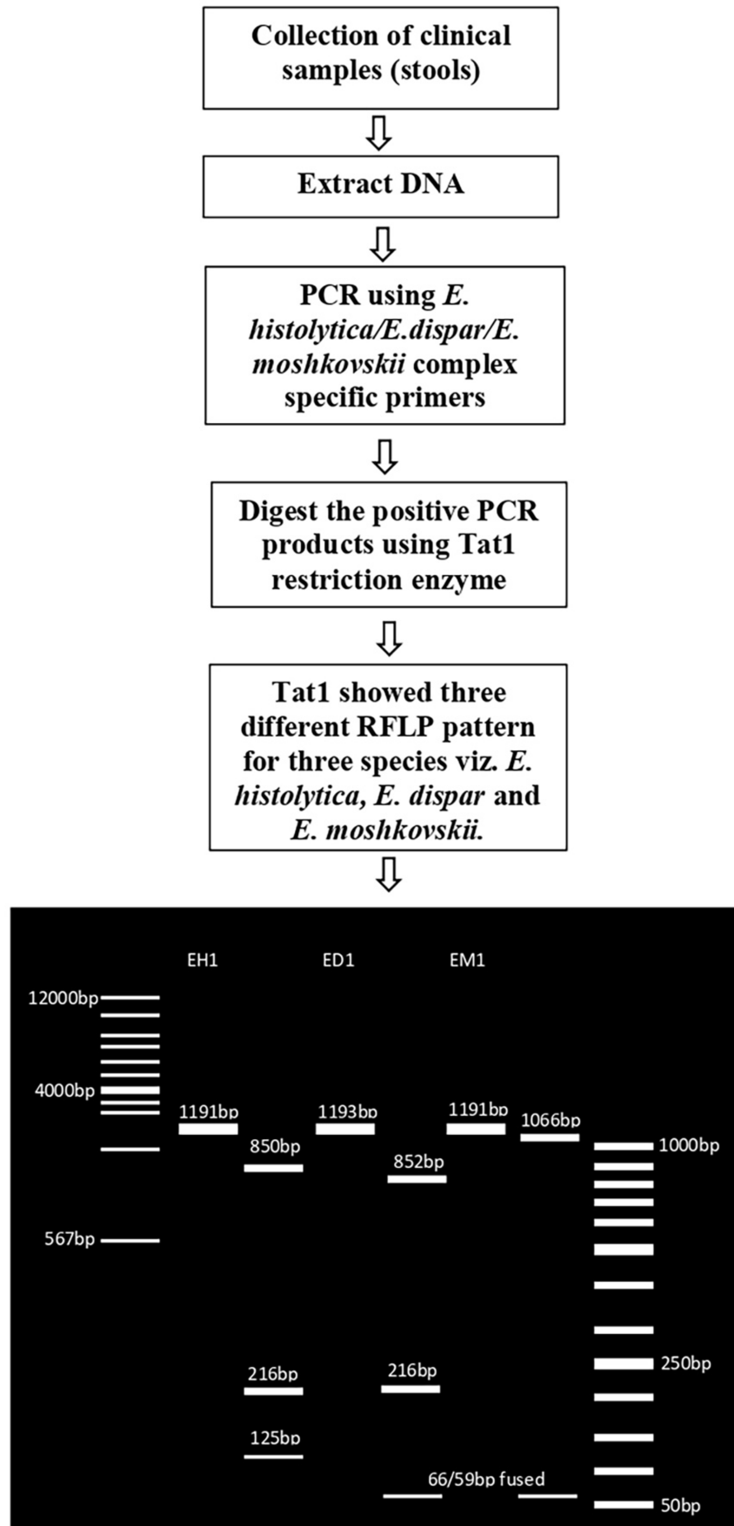


Fig. 9. 12 Flowchart showing the PCR-RFLP method developed for detection of *E. histolytica, E. dispar* and *E. moshkovskii.* EH1>Amplified PCR product of *E. histolytica* based on 18SrRNA locus, EH2> Restriction patterns (125 bp, 850 bp and 216 bp) of *E. histolytica*; Ed1>Amplified PCR product of *E. dispar* based on 18SrRNA locus, Ed2> Restriction patterns (66 bp/ 59 bp fused band, 852 bp and 216 bp) of *E. dispar*; EM1> Amplified PCR product of *E. moshkovskii* based on 18SrRNA locus, EM2> Restriction patterns (66 bp/ 59 bp fused band and 1066 bp) of *E. moshkovskii*.

9.4 Development of single-round multiplex PCR assay for differentiation of morphologically identical *Entamoeba* species

In the preceding section, we have discussed the development of an affordable and straightforward 18S PCR–RFLP technique to identify and distinguish the three clinically important *Entamoeba* species. Despite the exceptional specificity, sensitivity, and high reproducibility of the PCR-RFLP method, it is not well-suited for simultaneously detecting multiple *Entamoeba* species infections in a single individual, which refers to the co-infection of multiple *Entamoeba* species. To address the requirement for a specific diagnostic test to detect and differentiate *Entamoeba* species, we have also successfully designed and developed a single-round multiplex PCR assay system. This system is intended for use in both clinical and research settings, offering several advantages such as enhanced sensitivity and specificity. The multiplex PCR assay proves to be a valuable tool in identifying and distinguishing various *Entamoeba* species. Previous studies have primarily focused on employing nested multiplex PCR assays limited to detecting and differentiating *E. histolytica, E. dispar* and *E. moshkovskii*. Moreover, recent research highlights the clinical relevance of *E. bangladeshi*, which shares similar morphology with the aforementioned species. Additionally, certain studies have emphasized the notable clinical significance of *E. coli.* Consequently, there is a need for the development of improved multiplex PCR assays that encompass these additional species and take into account their clinical implications. Thus, this study endeavours to address this gap by developing a single-round multiplex PCR system capable of detecting and distinguishing five *Entamoeba* species: *E. histolytica, E. dispar, E. moshkovski, E. bangladeshi,* and *E. coli.* To the best of our knowledge, no such technique has been established thus far for the detection and differentiation of these five species from biological samples.

In this chapter section, we will examine the development and evaluation of an innovative single-round PCR assay technique. This technique aims to detect and distinguish *E. histolytica*, *E. dispar*, *E. moshkovskii, E. bangladeshi*, and *E. coli* simultaneously in clinical samples. It offers simplicity, conciseness, labor efficiency, and cost-effectiveness. This novel single round PCR assay technique has the potential to become an excellent tool for the molecular identification of *Entamoeba* species in diagnostic applications in the near future.

9.4.1 Methodology

9.4.1.1 Primer Design

The sequences of the following species were analyzed for primer design: *E. histolytica*, GenBank accession no. X56991 (1947 bp); *Entamoeba dispar*, GenBank accession no. Z49256 (1949 bp); *E. moshkovskii*, GenBank accession no. AF149906 (1944 bp); *Entamoeba bangladeshi*, GenBank accession no. KR025411 (1927 bp); *Entamoeba nuttalli*, GenBank accession no. AB485592.1 (2431 bp); *Entamoeba coli*, GenBank accession no. AF149914 (2101 bp); *Entamoeba chattoni*, GenBank accession no. AF149912 (1963 bp); *Entamoeba polecki*, GenBank accession no. AF149913 (1858 bp); *E. invadens*, GenBank accession no. AF149905 (1965 bp). The sequences were aligned using the ClustalW program (https://www.genome.jp/tools-bin/clustalw). We have developed a universal reverse primer that is shared among all five species. Additionally, we have designed species-specific primers for each individual species. The PCR amplification using these primers yielded distinct-sized products for each species, allowing for the verification of their identities in multiplex PCR system. When *E. dispar* is present, the expected PCR product size is 1373 bp. For *E. histolytica*, the expected size is 922 bp. *E. bangladeshi* produces a 764 bp amplicon, *E. moshkovskii* yields a 495 bp product, and the presence of E. coli results in an expected amplicon size of 204 bp.

To ensure specificity and exclude possible cross-hybridizations, we compared all primers with sequences in the GenBank database. We carefully considered the length and base composition of the primers to ensure they would have a common annealing temperature, enabling their simultaneous use. Furthermore, we conducted DNA sequencing of all PCR products to evaluate the specificity and sensitivity of designed primers in routine amoebic infection diagnosis.

Table 9.1 List of primers used in the multiplex PCR along with their melting temperature (Tm) values

Name of Primer	Sequence (5’-3’)	Tm
Ed/multi_F	GTACAAAGTGGCCAATTTATGT	55 °C
Eh/multi_F	GGTTTTATACATTTTGAAGACTTTATG	55.86 °C
Eb/multi_F	GACAATATTGAGGAGATGTCGTAAG	59.70 °C
Em/multi_F	CGGAAACCAAGAGTTTCACA	55.25 °C
Ec/multi_F	ATCATCTTCGGGTGGTTCTG	57.30 °C
EntaUniv_R	CCAAGATGTCTAAGGGCATCA	57.87 °C

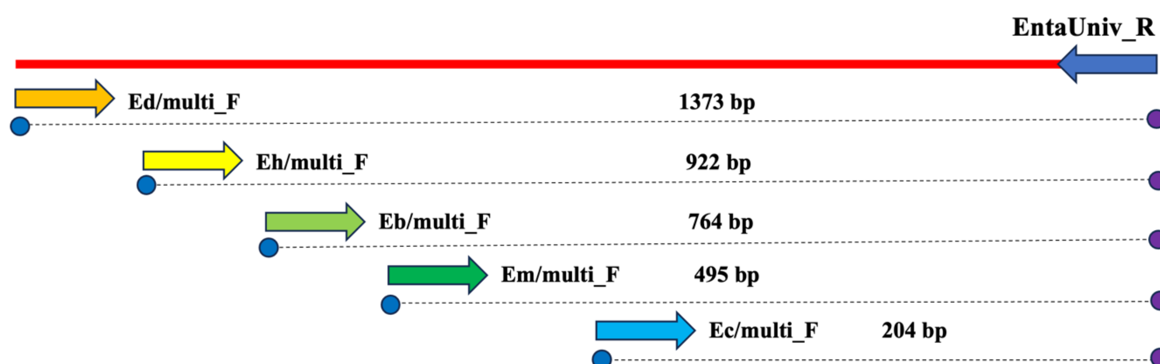


Fig. 9.13. Schematic representation of the design of Multiplex PCR, illustrating the positions of the primers utilized in this method.

9.4.1.2 DNA isolation

Genomic DNA of *E. histolytica* (HM1:IMSS clone 6) and *E. moshkovskii* (Laredo strain) were isolated directly from the routinely maintained axenic culture in our laboratory. The *E. moshkovskii* strain was a gift from Dr Seiki Kobayashi, Keio University, School of Medicine. DNA isolation was carried out using GENOMIC DNA Minikit (QIAGEN, USA) as per the manufacturer's protocol. The genomic DNA of *E. dispar* was also provided by Dr Seiki Kobayashi, School of Medicine, Keio University and the DNA was isolated as previously described. In order to ensure optimal conditions during the study, a positive control was included in each reaction. The positive control consisted of 5ng/µl of genomic DNA obtained from trophozoites in the logarithmic phase of growth for three species: *E. histolytica, E. moshkovskii,* and *E. dispar.*

Due to inadequate amounts of genomic DNA from both *E. bangladeshi* and *E. coli* samples, we employed rDNA plasmids as positive controls. Dr Yumiko Saito-Nakano, NIID, Japan has provided rDNA plasmids that contain DNA sequences of *E. bangladeshi* (KR025411.1) and *E. coli* (AF149914.1). These DNA sequences have been inserted into the pEx-A2J2 vector (Amp). The concentration of the provided plasmids is 0.1 µg/µL, which is equivalent to 19.2 x 10^14 copies of the plasmids per microliter. In the optimized multiplex PCR reaction, a final concentration of 0.0002 µg/µL of the plasmids containing *E. bangladeshi* and *E. coli* DNA sequences is used.

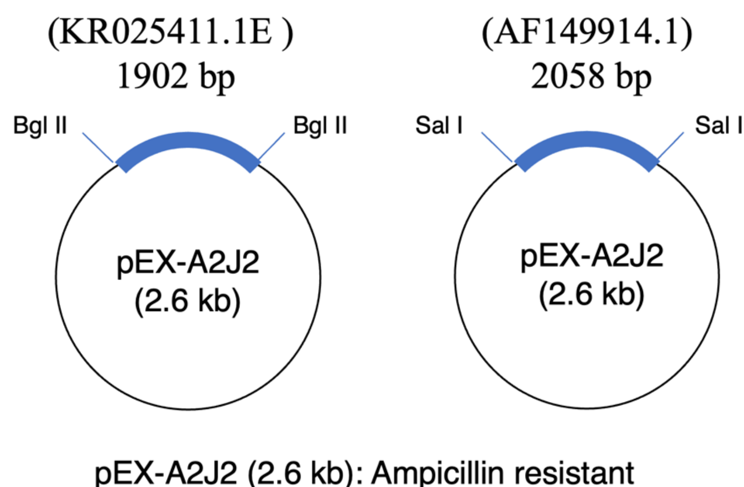


Fig. 9.14 rDNA plasmids used as positive controls for *E. bangladeshi* and *E. coli*.

9.4.1.3 PCR amplification

In order to achieve appropriate amplification of each target, adjustments were made to this single round multiplex PCR. The highest intensity of amplicons was observed at an optimum extension temperature of 72°C, surpassing any other temperature tested. The optimal concentration of MgCl2 was determined to be 3 mM after examining a range of concentrations from 1.0–4.0 mM. For optimal results, it was determined that a concentration of 0.2 µM for each primer (Ed/multi_F, Eh/multi_F, Eb/multi_F, Em/multi_F, Ec/multi_F and EntaUniv_R) was necessary. Additionally, the optimal annealing temperature was established as 55°C through the use of a gradient PCR. The amplification process was carried out in a thermal cycler using the following protocol: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles. Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 54°C for 25 seconds, and extension at 72°C for 40 seconds. A final extension step was performed at 72°C for 7 minutes. Subsequently, the PCR products were separated by electrophoresis using a 1.2% agarose gel and visualized by staining with ethidium bromide at a concentration of 0.5 µM/ml under a UV transilluminator.

9.4.1.4 Assessment of competition for non-target DNA

To evaluate the potential non-target amplification of the primer set, we utilized DNA extracted from stool samples known to be positive for *E. invadens, E. polecki, E. chattoni, Cryptosporidium parvum, Giardia lamblia*, as well as mixed bacterial infections. These samples were used as templates in the optimized reaction system.

9.4.1.5 Estimation of the minimum concentration of template DNA detectable by the single-round multiplex PCR

To assess the sensitivity of the primer set, we prepared serial dilutions of reference DNA templates for *E. histolytica, E. dispar, E. moshkovskii*. The sensitivity of the primer set was evaluated using reference DNA templates of the three *Entamoeba* species by two-fold serial dilutions from 10 to 0.0013 ng/µL of DNA. Each dilution was prepared using nuclease-free water.

Subsequently, PCR amplifications were performed according to the previously mentioned protocol using these diluted DNA templates. This allowed us to evaluate the sensitivity of the primer set by determining the lowest concentration at which the target species could still be detected.

We were unable to determine the minimum detectable concentration of DNA for *E. bangladeshi* and *E. coli* due to insufficient genomic DNA samples.

9.4.1.6 Evaluation using known positive stools DNA sample:

In chapters 3 and 4 of our surveillance study, we collected a significant number of positive samples for *E. histolytica, E. dispar,* and *E. moshkovskii*. In order to evaluate the efficiency of the multiplex PCR assay, we selected 10 samples that tested positive for *E. histolytica* and 10 samples that tested positive for *E. moshkovskii* from our previous surveillance study. Furthermore, we conducted an evaluation of the assay by testing five samples of amoebic liver abscess. These samples had previously been confirmed using PCR techniques, as outlined in Chapter 8 of our study. Additionally, to validate the multiplex PCR system, we included 8 samples that tested positive for *E. dispar* from the pool of positive samples obtained during the surveillance study. The amplification efficacy of multiplex PCR was evaluated by testing positive samples under optimized PCR conditions. The method was further validated by directly sequencing the amplified PCR products using Sanger sequencing.

9.4.2 Results

In our current study, we successfully utilized a specific primer set designed for the *E. histolytica, E. dispar, E. moshkovskii*, E. Bangladeshi and E. coli. These primers effectively generated the desired PCR products when tested with reference genomic DNA or rDNA (for *E. bangladeshi* and *E. coli*) (**Fig. 9.15**). Through a sensitivity assay, we demonstrated that the primers could amplify as little as 0.0049 ng/4.9 pg of genomic DNA from *E. histolytica*, *E. dispar,* and *E. moshkovskii,* as evidenced by visible bands with intensities exceeding 20 ng/µl. However, we encountered difficulties in evaluating the sensitivity of the primers specifically for *B. bangladeshi* and E. coli. Nonetheless, when rDNA from these species was present, both sets of primers successfully produced high-intensity visible bands with the expected amplicon sizes. This outcome suggests that the primers are effective for *E. bangladeshi* and E. coli, similar to their performance with the other target species. No non-specific amplification was detected when analyzing stool DNA extracted from positive samples containing *E. coli, E. invadens, E. polecki, E. chattoni*, *Cryptosporidium* sp., *Giardia lamblia*, and mixed bacterial infections. The primers designed in this study demonstrated efficient identification of individual Entamoeba species even when their DNA was mixed together, as shown in **Fig. 9.16**. Hence, this method is well-suited for screening multiple *Entamoeba* species in cases of coinfection.

Additionally, the surveillance study successfully amplified 18S rDNA fragments in previously identified *Entamoeba* species, including *E. histolytica, E. dispar,* and *E. moshkovskii*, using a multiplex PCR approach. To validate the results, the amplified PCR products were directly sequenced using Sanger sequencing and subsequently confirmed through comparison with the NCBI-BLAST database. The sequencing outcomes were consistent with the findings from the multiplex assay.

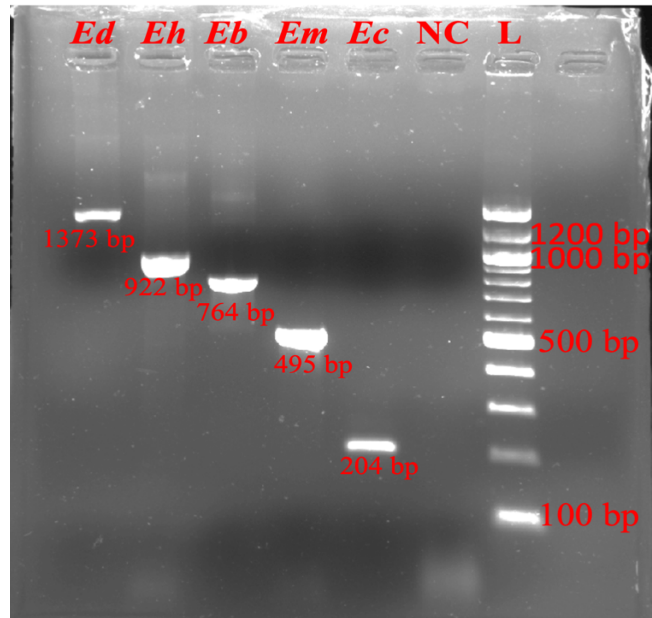


Fig. 9.15 Agarose gel electrophoresis image of a multiplex pcr assay for the detection of *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii, Entamoeba bangladeshi,* and *Entamoeba coli*. The gel image displays the results of a multiplex PCR assay targeting the presence of various *Entamoeba* species. The bands are organized into labelled lanes, with "Ed" representing *E. dispar*, "*Eh*" representing *E. histolytica*, "Eb" representing *E. bangladeshi*, "Ec" representing *E. coli*, "Nc" representing the negative control, and "L" indicating the 100bp DNA ladder (NEB). The presence of specific bands in each lane provides information about the presence of the respective *Entamoeba* species.

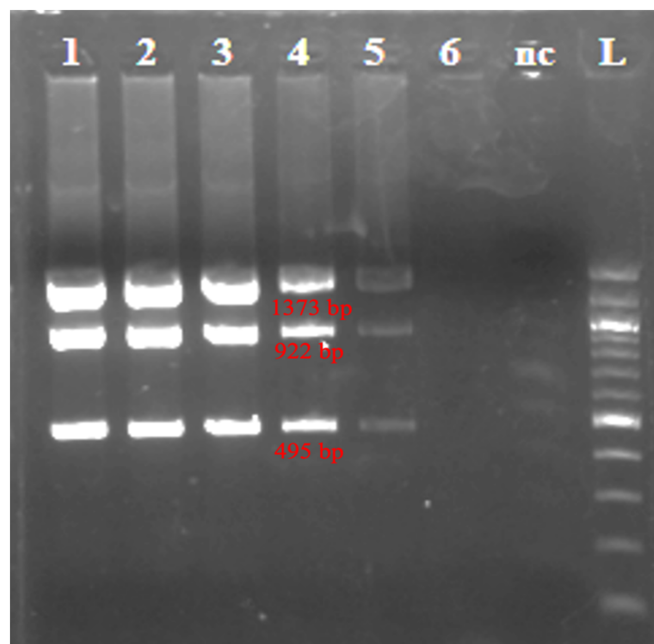


Fig. 9.16 Evaluation of detection specificity and sensitivity of co-infected *Entamoeba* species. Each reaction was performed by mixing equal amounts of genomic DNA from *E. diapar, E. histolytica,* and *E. moshkovskii.* Lane 1 contained 3 ng/µL, Lane 2 contained 0.6 ng/µL, Lane 3 contained 0.12 ng/µL, Lane 4 contained 0.024 ng/µL, Lane 5 contained 0.0048 ng/µL, and Lane 6 contained 0.00096 ng/µL of each genomic DNA. Nc: negative control. L : 100bp DNA ladder (NEB).

9.4.3 Discussion

The identification and characterization of *Entamoeba* species in epidemiological studies of amoebic infection pose challenges due to their similar morphological characteristics within the human intestine. Furthermore, the presence of cysts from other species like Iodamoeba or *Endolimax* can complicate the diagnosis process. To address these issues, a new approach was developed in the current study. A single-round multiplex PCR assay was created to detect and differentiate between *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. bangladeshi*, and *E. coli*. **(Fig. 9.15).** The developed assay offers a simple, cost-effective, and efficient approach that holds potential in the clinical diagnosis of amoebiasis.

The choice of targeting the 18S rRNA locus is advantageous due to its high conservation (Fontecha et al. 2015) and abundant copy number (approximately 200 copies per haploid genome), with no variations among the copies (Bhattacharya and Bhattacharya, 2013). This ensures both sensitivity and specificity of the assay. With the ability to analyze biological samples such as stools and liver abscesses, this technique facilitates the accurate identification of *Entamoeba* species. Furthermore, the distinct differences observed in the five bands allow for easy differentiation after agarose gel electrophoresis, enabling species identification. Compared to other techniques such as qPCR, ELISA, and individual single-round PCR, this multiplex approach is both time-saving and cost-effective. However, it is important to note that one major drawback of this technique is the requirement for higher expertise and skills during the preparation of the master mix. The susceptibility to contamination and the need for precise optimization make multiplex PCR potentially vulnerable to non-specific amplification. However, despite these drawbacks, the developed technique exhibits remarkable reproducibility under optimized conditions and requires minimal instrumentation. Consequently, this rapid technique holds promise for future diagnostic applications.

9.5 Development of a TaqMan probe-based multiplex qPCR assay for simultaneous detection of enteric parasites viz. *Giardia spp, Entamoeba spp* (*E. histolytica, E. dispar and E. moshkovskii) and Cryptosporidium* spp.

In diagnosing intestinal parasitic infections, ELISA exhibits higher sensitivity compared to microscopic detection techniques. However, when compared to DNA-based assays, ELISA is found to be less sensitive and specific. (Delialioglu et al. 2008). DNA amplification assays, such as polymerase chain reaction (PCR), have demonstrated remarkable sensitivity and specificity compared to other molecular techniques a (Blessmann et al. 2002). Within this chapter, two diagnostic methods have been introduced: the 18S PCR-RFPLP assay and the multiplex technique for diagnosing amoebic infections. Each method possesses its unique advantages and limitations. These methods available for detection only target clinically significant species of *Entamoeba*. However, there are two other frequently encountered groups of enteric parasites associated with diarrheal cases, namely *Giardia lamblia* and *Cryptosporidium* spp. Hence, there is a critical need for a highly sensitive and specific molecular technique that can rapidly identify all species of these parasites in a single platform.

Conventional/nested PCR is a highly sensitive method for detecting and differentiating enteric parasites, but it is known to be a time-consuming procedure (Souza et al. 2007). Nested PCR assays are known for their exceptional sensitivity but are also prone to contamination. While multiplex PCR assays can be valuable for detecting and distinguishing various enteric parasites, standardizing the technique can be challenging, and non-specific amplifications are a significant drawback. PCR-RFLP technique is not ideal for diagnosing cases with multiple co-infections. In endemic areas, test centers relying on multiplex PCR techniques must either standardize existing protocols or develop their own to ensure accurate differentiation of enteric parasites (Fontecha et al. 2015). Although molecular-based methods for detecting enteric parasites have demonstrated good sensitivity and specificity, implementing these parasite-specific methods in routine diagnostic laboratories can be time-consuming and requires significant manpower (Sow et al. 2010). Consequently, there is a need for a more convenient diagnostic method that enables rapid and reliable detection of all parasites associated with diarrheal cases in a single platform.

qPCR techniques offer several advantages, including the ability to detect low levels of parasites, enhance the identification of infected individuals, and assess the effectiveness of treatments through quantification. Real-time PCR is particularly advantageous when compared to

conventional PCR, multiplex PCR, and PCR-RFLP approaches. This methodology reduces the risk of contamination and saves time. Additionally, the probe-based qPCR technique demonstrates greater sensitivity compared to other DNA-based molecular methods.

In this section, a Taqman-based multiplex qPCR assay was developed and evaluated for the diagnosis of parasitic diseases. The method consisted of two separate reactions. In the first reaction, the goal was to identify *Giardia lamblia*, *Entamoeba* spp (including *E. histolytica*, *E. moshkovskii*, and *E. dispar*), and *Cryptosporidium* spp. In the second round of reaction, the three most clinically significant *Entamoeba* species, namely *E. histolytica, E. dispar*, and *E. moshkovskii*, were differentiated using species-specific probes.

9.5.1 Methodology

9.5.1.1 Ethics review

Stool samples will be obtained from different hospitals and from local rural and urban areas as per the Institutional Review Board, in compliance with their ethical guidelines. In accordance with the Institutional Human Ethics Committee written informed consent from the patient will be taken (in the case of children, consent will be obtained from their parents).

9.5.1.2 Analyzing of Gene Regions and Designing of Primers and Probes

In this research, the 18S rRNA and β-Giardin loci were the focus of investigation. The goal was to identify genetic polymorphisms in these loci and develop specific primers and probes. To achieve this, various regions of the 18S rRNA/β-Giardin loci were analyzed through in silico methods, taking into account thermodynamic conditions. A combination of bioinformatics tools was utilized, including CLC Genomics Workbench 12 (CLC, Bio-QIAGEN, Aarhus, Denmark), Allele ID, and Gene Runner 6.5.52 software. Reference sequences were collected from GenBank and imported into the CLC Genomics Workbench 12 software for analysis and further processing. The most suitable regions were chosen for the development of a TaqMan-qPCR assay. To optimize the melting temperature (Tm) of the primers and probes, predictions from OligoAnalyser 3.1 software were taken into consideration. Specifically, the 18S rRNA region was selected for the identification of *Entamoeba* spp and *Cryptosporidium* spp, while the β-Giardin region was chosen for the identification of *Giardia* spp. The Primer-Blast tool was utilized to assess the specificity of the designed primers and probes for all genes.

To construct an internal control, the RNAse P gene structure was analyzed, and a probe was designed specifically for this purpose. The characteristics of the probes used in the first reaction for the detection of *Giardia* spp, *Entamoeba* spp, and *Cryptosporidium* spp included the reporter dyes FAM, ROX, and HEX, along with the BHQ1, BHQ2, and BHQ1 quenchers, respectively. For the second reaction, aimed at differentiating *Entamoeba* species such as *E. histolytica, E. dispar,* and *E. moshkovskii*, the probes were designed with the reporter dyes HEX, ROX, and FAM, and the BHQ1, BHQ2, and BHQ1 quenchers, respectively (**Table 9.2**). The procurement of primers (**Table 9.4** and **Table 9.5**) and probes (for details refer **Fig. 9. 17 a. and b.**) was done from Eurofins and GenScript, located in the USA, respectively.

Table 9.2 Fluorescence reporters and detectors used for designing the qPCR assay.

Fluorescence reporters and detectors

Target	Target Gene	Reporter	Quencher
***Giardia* sp**	β-Giardin	FAM	BHQ1
***Entamoeba* sp**	18S rRNA	ROX	BHQ2
***Cryptosporidium* sp**	18S rRNA	HEX	BHQ1
Human Internal Control	RNAse P	Cy-5	BHQ2

Target	Target Gene	Reporter	Quencher
Entamoeba histolyitica	18S rRNA	HEX	BHQ1
Entamoeba dispar	18S rRNA	ROX	BHQ2
Entamoeba moshkovskii	18S rRNA	FAM	BHQ1
Human Internal Control	RNAse P	Cy-5	BHQ2

Table 9.4. Primers and probes used for used for detection of the enteric parasites in the developed qPCR assay.

Organism	Primer	Probe
Entamoeba spp.	5'-CGTGGCAATGACGGGTAA-3' (EntaF)	5'-HEX-TTAGGGTTCGATTCCGGAGAGGGA-BHQ1-3'
	5'-TGCTGCCTTCCTTAGATGTG-3' (EntaR)	
Giardia spp.	5'-CTTACCAAGACCGAACAGTAGAA-3 (GiaF)	5'-ROX-TAGTGGTGCATGGCCGTTCTTAGT-BHQ2-3'
	5'-CCTGACAAATCACTCCACCA-3'(GiaR)	
Cryptosporidium spp.	5'-GCCGACAACATGTACC-3' (CryptF)	5'-FAM-ACGATCAAGGAGGAGATCGACACCATGGCBHQ1-3'
	5'-GACTTGCGGAAGTTTGC-3'(CryptR)	

Table 9.5 Primers and probes used for used for detection of different *Entamoeba* spp. in the developed qPCR assay.

Organism	Primer	Probe
E. histolytica	5'-CAGTAATAGTTTCTTTGGTTAGTAAAA-3' (EhqF)	5'-HEX-GTTTGTATTAGTACAAAATGGC-BHQ1-3'
	5'-CTTAGAATGTCATTTCTCAATTCAT-3' (EhqR)	
E. dispar	5'-CAGTAATAGTTTCTTTGGTTAGTAAAG-3'(EdqF)	5'-ROX-GTATTAGTACAAAGTGGCCAA-BHQ2-3'
	5'-CTTAGAATGTCATTTCTCAATTTAC-3'(EdqR)	
E. moshkovskii	5'-CAGATGGCTACCACTTCTAC-3' (EmqF)	5'-FAM-CTCGAGGTGGTTAACTCCAC-BHQ1-3'
	5'-GATTTCGTAAGAGTATTTACTTCT-3'(EdqR)	

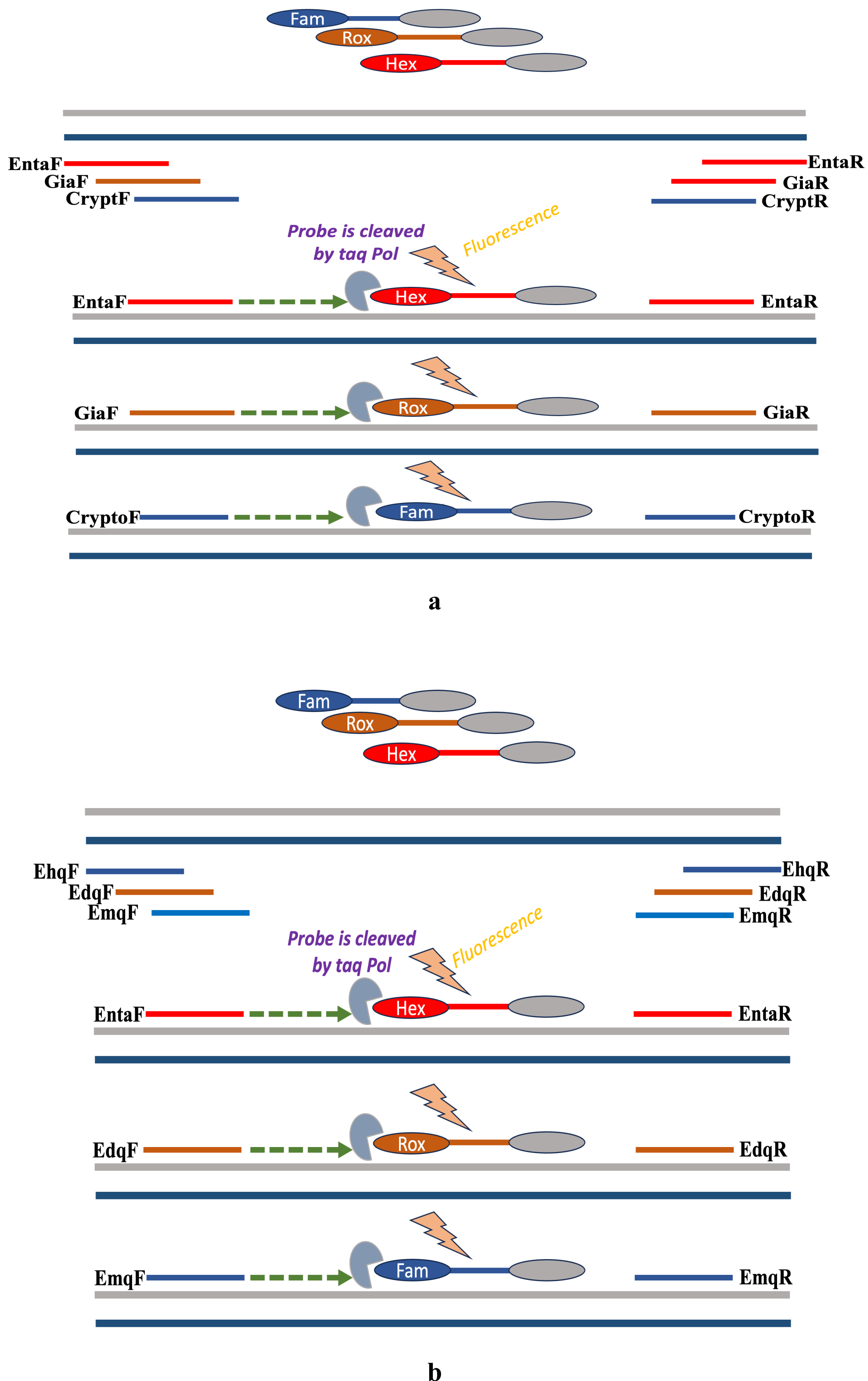


Fig. 9. 17 a. and **b.** Design of multiplex qPCR for detection of common enteric parasites identification. EntaF and EntaR for *Entamoeba* spp identification. GiaF and GiaR for *Giardia* identification. CryptoF and CryptoR for *Cryptosporidium* identification. EhqF and EhqR for *E. histolytica* identification. EdqF and EdqR for *E. dispar* identification. EmqF and EmqR for *E. moshkovskii* identification.

9.5.1.3 DNA isolation

The DNA was isolated from the test stool samples using STOOL DNA Minikit (QIAGEN, USA) as per manufacturer's protocol. DNA from Liver aspirates of Amoebic Liver Abscess (ALA) were isolated using DNeasy Blood & Tissue Kit (QIAGEN, USA). The purity of the extracted DNA was evaluated and will only be accepted if the OD value at 260\280 is approximately 1.8. After isolation, the DNA was kept under refrigeration (- 20°C) for future analysis by PCR. Reference DNA for Entamoeba and *Giardia* was collected from axenic culture and *Cryptosporidium* reference DNA was collected from previously confirmed stool samples screened by acid-fast staining and conventional PCR. The NanoDrop 2000c spectrophotometer (Thermo Scientific, Asheville, NC, USA) was utilized to quantify the purified DNA.

9.5.1.4 Developing the Multiplex qPCR Quantification

In our multiplex qPCR study, we prepared a 20 µl reaction mix for each sample. The reaction mix included the following components: 3 µl of template DNA at a concentration of 10 ng/reaction, 5 µl of reaction buffer mix (BBL) for the Probe without ROX, 0.4 pmol of each primer (Eurofins), and 0.3 pmol of each probe (Eurofins) (refer **Table 9.4** for more details).

We conducted the amplification process using a BioRad Real-Time PCR Detection System. The cycling conditions were as follows: an initial denaturation step at 95°C for 3 minutes, followed by 40 amplification cycles. Each cycle consisted of denaturation at 95°C for 10 seconds, followed by annealing and extension at 60°C for 30 seconds. In each experiment, a negative control was included where no template DNA was added. Additionally, each DNA sample was assessed in triplicate. Any samples that had a cycle threshold (CT) value greater than 35 were deemed negative (**Table 9.5**).

Table 9.4 Optimized Protocol for the Detection of Common Enteric Parasites Using TaqMan Probe-Based Multiplex PCR

Component	Volume per reaction		
	Sample Reaction	Positive Control Reaction	Negative Control Reaction
Enzyme (BBL)	1 µL	1 µL	1 µL
Reaction Buffer Mix (BBL)	5 µL	5 µL	5 µL
Multiplex Probe and Primer Mix (Eurofins)	6.38 µL	6.38 µL	6.38 µL
DNA Sample	3 µL	-	-
Positive Control*	-	2 µL	-

Nuclease Free Water	4.62 µL	5.62 µL	7.62 µL
Total Volume	20 µL	20 µL	20 µL

- **The reaction setup adhered to the following guidelines:**

✓ *Prepare the whole reaction on ice (or in a cold room) and keep the reaction plate on ice (or in a cold room) until it is loaded into the real-time PCR instrument.*

✓ *Run the plate after preparation. Prolonged storage could result in the degradation of DNA samples.*

✓ *To prevent contamination, do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.*

✓ *Maintain a nuclease-free environment.*

✓ *Protect assays from light as the probes supplied in the kit are light sensitive.*

✓ *For each PCR plate, include one positive control and one negative control along with the patient DNA samples.*

Table 9.5 Standardized PCR Program obtained from preliminary study

	Stage	Cycle Repeats	Acquisition / Data Collection	Temperature	Time Min:Sec
Initial Denaturation	**Hold**	**1**	**-**	**95°C**	**03:00**
Amplification	**Cycling**	**40**	**-**	**95 °C**	**00:10**
			Yes	**60 °C**	**00:30**

9.5.1.5 Specificity Testing of the qPCR Assay

To assess the specificity of the developed triplex qPCR assay, various DNA templates were utilized, including *Cyclospora* spp, *Paragonimus westermani*, *Ascaris lumbricoides*, *Entamoeba coli*, DNA from a mixed bacterial infection such as *C. jejuni*, *C. coli,* and *Schigella* sp. Sterile distilled water served as the blank control, while normal human fecal DNA was used as the negative control. Genomic DNA of *E. histolytica, E. moshkovskii*, and *G. lamblia* was employed as the positive control in the specificity test.

9.5.1.6 Testing previously known Patients Samples Using the qPCR assay

In the conducted study, a total of 64 stool samples were examined for the presence of enteric parasites, specifically *Entamoeba spp, G. lamblia,* and *Cryptosporidium* spp. The detection and confirmation of these parasites were carried out using microscopy and conventional PCR assays, as described in Chapter 3 and 4 of the study.

Among the samples, two were found to be mixed infections of different *Entamoeba* spp, while one sample exhibited a mixed infection of *G. lamblia* and *Cryptosporidium* spp. These mixed infections were further evaluated using a developed qPCR method. The samples were collected from the surveillance study conducted at ID Hospital and BC Roy Children Hospital in Kolkata. Detailed information regarding the sample collection can be found in the earlier chapter (Chapter 3) of the study. Additionally, five liver aspirates from patients with amoebic liver abscesses (ALA) were included in the study. These ALA samples were personally collected from various hospitals in Kolkata as part of a collaborative research endeavour. The protocols for this study were approved by the Institutional Human Ethics Committee of ICMR-NICED. To determine the specificity of the developed methods, a total of 83 negative clinical samples (for parasites) were also included in the validation process.

9.5.1.7 Limit of Detection (LOD) of the qPCR Assays

The Limit of Detection (LoD) of this kit determines the lowest detectable concentration of enteric parasites present in stool DNA samples, at which approximately 95% of all true positive replicates test positive. The LoD of this kit was determined using a reference DNA control. Reference genomic DNA was extracted from axenic cultures of *E. histolytica*, *E. moshkovskii*, and *G. lamblia*. To create a range of DNA concentrations for analysis, serial dilutions were performed using 10-fold dilutions from 10 to 0.000001 ng. Amplification reactions were carried out under optimized conditions. The concentration of beta-giardin was quantified by determining the number of copies in the genome, calculated based on the weight of a single *Giardia lamblia* genome. *G. lamblia* has an estimated genome size of 11.7 Mb (as reported in Jerlström-Hultqvist J et al. 2010). Approximately $7.92\text{x}10^{5}$ copies of genomes are present in 10 ng of *G. lamblia* genomic DNA. For very low concentrations, such as 0.000001 ng of *G. lamblia* genomic DNA, the approximate number of genome copies is $7.92\text{x}10^{-2}$. Regarding Entamoeba and *Cryptosporidium*, we targeted the 18S rRNA locus for analysis. However,

determining the concentration of 18S rRNA is challenging due to its variable presence in the genome. During the determination of the Limit of Detection (LOD) for these species, we focused on calculating the number of genome copies present in the given amount of DNA. The average genome sizes for *E. histolytica, E. dispar*, and *E. moshkovskii* are reported to be 20.8 MB, 23 MB, and 25.2 MB, respectively (Wilson et al. 2019). In 10 ng of *E. histolytica, E. dispar,* and *E. moshkovskii* genomic DNA, there are approximately 4.45×10^5, 4.03×10^5, and 3.68×10^5 copies of genomes, respectively. 0.000001 ng of genomic DNA from *E. histolytica*, *E. diaper*, and *E. moshkovskii* contains approximately 4.45×10^{-2}, 4.03×10^{-2}, and 3.68×10^{-2} copies of genomes, respectively. Standard curves were created by serially diluting this DNA to establish the limits of detection (LOD) and for absolute quantitation of DNA from stool samples. To assess reproducibility (inter-assay variability), a control DNA sample was amplified in five separate reactions.

To assess and establish precision, validations were conducted by running three replicates of each standard on two separate machines. The precision analysis involved calculating the threshold cycle (CT) values obtained from both devices. Inter-assay validation was performed to evaluate the consistency between the two machines, while intra-assay validation assessed the reproducibility within each machine.

9.5.1.8 Statistical Analysis

The data from the above study was analyzed by GraphPad Prism 8.0.

9.5.2 Results

To establish a consistent limit of detection, a threshold value of Ct = 35 was set for all primer sets and probes utilized in the analysis. This standardized cut-off ensured uniformity across the detection process. The qPCR assay design demonstrated 100% specificity, as no amplification was observed when testing other genomic DNA samples, including *Cyclospora* spp, *Paragonimus westermani*, *Ascaris lumbricoides*, *Entamoeba coli*, DNA from a mixed bacterial infection such as *C. jejuni*, *C. coli*, and *Schigella* sp. The sensitivity of the assay for detecting *E. histolytica* and *E. moshkovskii* was very high, with a detection limit as low as 3 fg and CT values ranging from 33 to 35. For *G. lamblia*, the detection limit of the assay was 30 fg, again with CT values ranging from 33 to 35.

The performance of the qPCR assay was evaluated beyond the detection limit concentration, revealing inconsistent and CT values above 35. Unfortunately, due to a lack of pure genomic DNA samples, we were unable to determine the limit of detection for the qPCR assays targeting *Cryptosporidium* spp and *E. dispar*. However, when serially diluting stool DNA samples that were positive for *Cryptosporidium* spp and *E. dispar* based on microscopy and conventional PCR, the qPCR assay produced expected results with varying CT values, indicating the effectiveness of the assay. The CT values ranged from low to high, reflecting the concentration gradient of the target DNA in the samples.

In a blinded evaluation, a newly developed multiplex PCR assay was tested against a collection of 64 samples. These samples had previously been examined using microscopy and conventional PCR to identify parasites. Among the samples, 62 were positive for at least one type of parasite, while 2 samples exhibited mixed infections. The results of the developed quantitative PCR (qPCR) revealed that 32 samples were positive for Giardia spp, 15 samples were positive for *E. moshkovskii*, 1 sample was positive for E. dispar, 5 samples were positive for *E. histolytica*, and 9 samples were positive for *Cryptosporidium* spp. Additionally, two samples showed mixed infections, one with *Giardia* and *Cryptosporidium*, and another with *E. histolytica* and *E. moshkovskii*. Importantly, the findings obtained through the qPCR assay were in complete agreement (100% consistency) with the results obtained from PCR and microscopy screenings. Upon conducting blind screening using qPCR, it was discovered that all five samples of amoebic liver abscess (ALA) were positive for *E. histolytica*. These particular ALA samples had previously tested positive for *E. histolytica* through PCR analysis. Out of 83 samples that tested negative using PCR and microscopy, the qPCR results showed one sample positive for *E. moshkovskii* and one sample positive for *G. lamblia*. These two samples tested negative during triplicate microscopy and conventional PCR testing. These results suggest that our qPCR technique is 2.41% more sensitive compared to PCR and microscopy. However, it is important to note that a more comprehensive study is required to draw a definitive conclusion. It should be remembered that we only conducted a pilot study using the newly developed qPCR assay.

Fig 9.20 to 9.21 showcases a series of experiments conducted as part of the evaluation process for our newly developed qPCR assay.

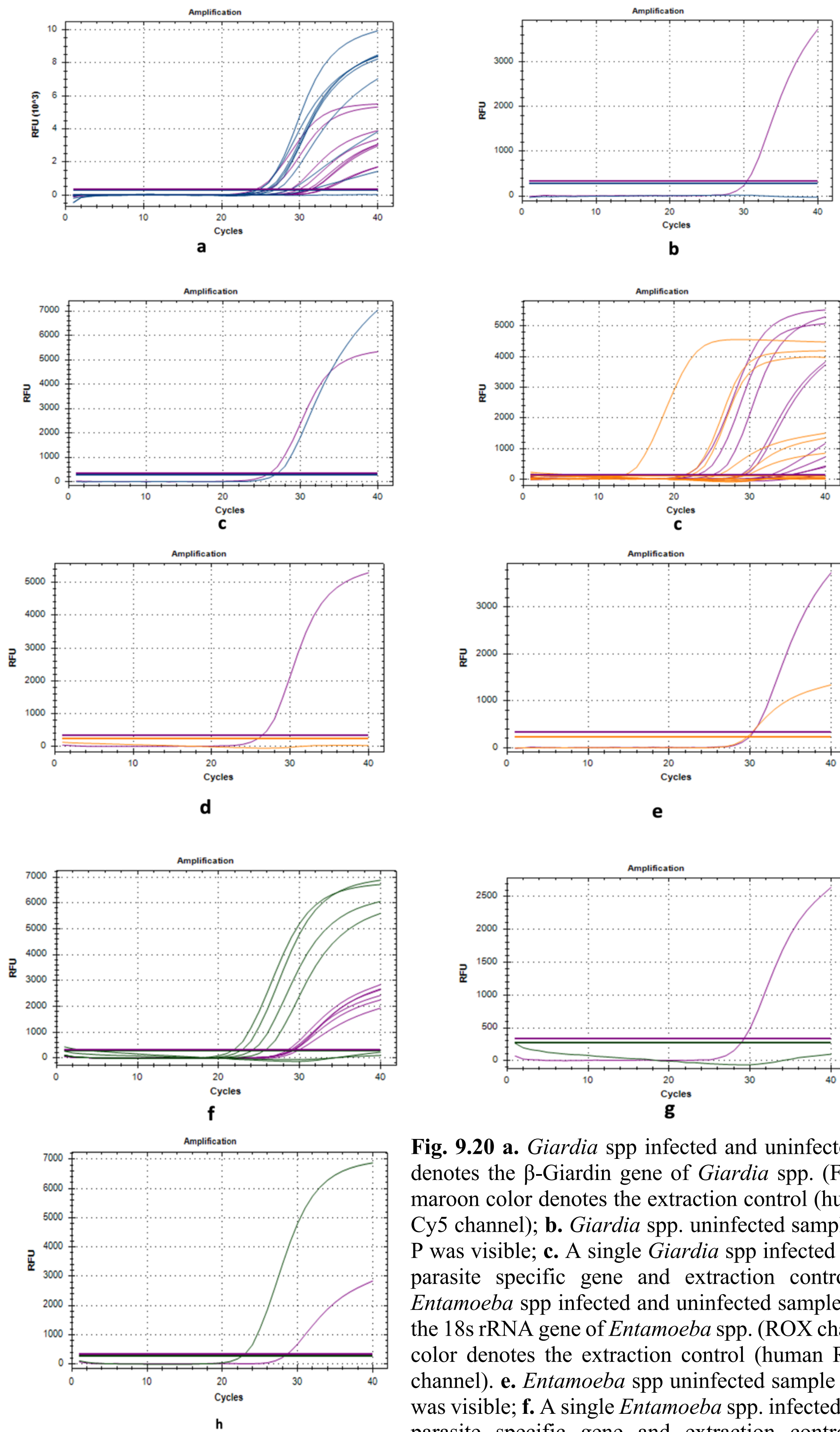


Fig. 9.20 a. *Giardia* spp infected and uninfected samples; blue color denotes the β-Giardin gene of *Giardia* spp. (FAM channel) whereas maroon color denotes the extraction control (human RNAse P gene in Cy5 channel); **b.** *Giardia* spp. uninfected sample where only hRNAse P was visible; **c.** A single *Giardia* spp infected sample where both the parasite specific gene and extraction control were observed. **d.** *Entamoeba* spp infected and uninfected samples orange color denotes the 18s rRNA gene of *Entamoeba* spp. (ROX channel) whereas maroon color denotes the extraction control (human RNAse P gene in Cy5 channel). **e.** *Entamoeba* spp uninfected sample where only hRNAse P was visible; **f.** A single *Entamoeba* spp. infected sample where both the parasite specific gene and extraction control were observed; **g.** *Cryptosporidium* spp. infected and uninfected samples orange color denote the 18s rRNA gene of *Cryptosporidium* spp. (HEX channel) whereas maroon color denotes the extraction control (human RNAse P gene in Cy5 channel); **h.** *Cryptosporidium* spp. uninfected sample where only hRNAse P was visible; **i.** A single *Cryptosporidium* spp infected sample where both the parasite specific gene and extraction control were observed.

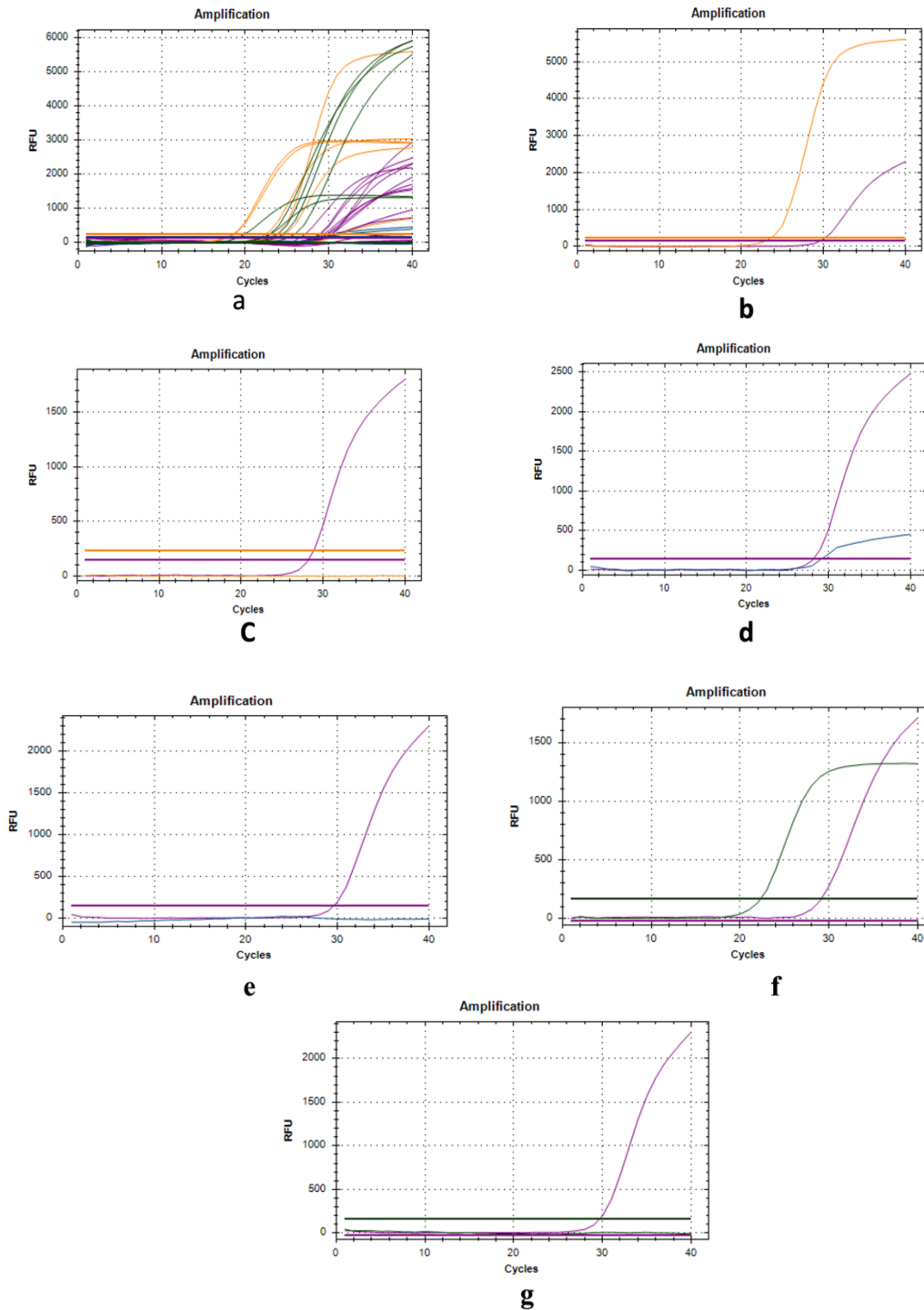


Fig. 9.21 All the *Entamoeba* positive samples were analyzed in this multiplex PCR, **a.** *Entamoeba histolytica* (HEX channel/ Green), *Entamoeba dispar* (ROX channel/ Orange) & *Entamoeba moshkovskii* (FAM channel / Blue) all 3 infected samples were visualized, **b.** Single *E. dispar* infected sample with hRNase P in Cy5 channel **c.** *E dispar* negative sample where only hRNase P were visualized, **d.** Single *E. moshkovskii* infected sample with hRNase P in Cy5 channel. **e.** *E moshkovskii* negative sample where only hRNase P were visualized. **f.** Single *E. histolytica* infected sample with hRNase P in Cy5 channel g. *E histolytica* negative sample where only hRNase P were visualized NTC (No template control) sample was also run simultaneously in each run however data was not shown in these graph.

9.5.3 Discussion

In the study, we devised a rapid and highly sensitive method to simultaneously identify morphologically identical yet clinically significant *Entamoeba* species, along with *Giardia* and *Cryptosporidium* species. These parasites are prevalent causes of diarrheal diseases. Additionally, we found that a portion of the supposed "*E. histolytica* infections" were actually caused by morphologically indistinguishable *Entamoeba* species like *E. dispar* and *E. moshkovskii*. In this study, our primary aim was to devise a detection method capable of specifically and exclusively identifying *Entamoeba* species. Our qPCR assay demonstrated comparable performance to conventional PCR and microscopy when applied to cultured and liver abscess DNA. Moreover, it exhibited improved sensitivity when analyzing stool DNA, successfully detecting additional cases of *E. moshkovskii* and one additional case of *G. lamblia* that conventional PCR and microscopy methods failed to identify. We developed the highly sensitive qPCR method that can detect Entamoeba DNA at less than one trophozoite per reaction, with the ability to identify DNA from just 0.1 trophozoite equivalent. This high sensitivity is due to the presence of multiple copies of the target 18S small-subunit ribosomal rRNA gene molecules in each amoeba/*Cryptosporidium* genome. However, the sensitivity of our qPCR method for *Giardia* is nearly 10 times lower compared to *Entamoeba* and *Giardia*. This is because we targeted the Beta-giardin gene, which exists as a single copy per *Giardia* genome. The qPCR method offers several advantages for detecting *Giardia*, one of which is its high sensitivity. Additionally, it benefits from using smaller amplicon sizes, making the amplification process easier. Another crucial factor is that during stool DNA isolation, DNA fragmentation may occur. However, when the targeted region is small, the chances of successful detection are significantly increased. A different qPCR technique was created by Ali and Roy, 2020. However, it was not specifically designed for detecting *Giardia* and *Cryptosporidium*. They only focused on detecting *Entamoeba* species. Our method, on the other hand, can effectively identify all common enteric parasites in one platform.

Before the COVID era, real-time PCR machines were not commonly used in diagnostic labs in developing countries. However, since the pandemic outbreak, these machines have become more widely available and are now commonly used in both private and government diagnostic laboratories. This makes it easier to implement this technique in developing countries like India.

The *Entamoeba* qPCR test has certain limitations that should be considered. In cases where there is a coexisting parasitic infection and the DNA from another species substantially surpasses the amount of *Entamoeba* DNA (e.g., over 150 times higher), the qPCR test will primarily detect and identify the dominant species. Consequently, the presence of *Entamoeba* might be masked or overlooked in such mixed infections. Another aspect to be mindful of is when dealing with formalin-fixed samples. These samples may experience compromised results due to formalin-induced DNA degradation, which can interfere with the accurate detection of *Entamoeba*. This degradation can lead to false-negative outcomes, where the test might fail to identify the presence of *Entamoeba* even if it is present in the sample.

Based on genomic DNA isolated from a pure culture, we have determined the LOD. However, we were unable to ascertain the minimum detection limits in stool samples. Therefore, it is uncertain whether the results obtained from clinical samples would be as accurate as our performed LOD assay. In addition, we have assessed the assay on a limited number of stool samples, which prevents us from accurately determining the sensitivity of the technique. To properly evaluate our method, it is necessary to screen a significant number of clinical samples from various geographical areas. After assessing the advantages and disadvantages, it seems that qPCR has great potential for use in epidemiological studies, research, diagnosis, and management of enteric parasites that cause diarrhoea. We have developed a new multiplex PCR and a PCR-RFLP approach in addition to the qPCR, which enables us to accurately detect and distinguish between *Entamoeba* species that look identical. Each technique has its own advantages and disadvantages. Selecting the appropriate method to identify and study parasites is crucial and depends on the intended purpose, available resources, and facilities.

9.6 Conclusion

We have successfully developed three distinct diagnostic methods for detecting medically significant *Entamoeba* spp.:

PCR-RFLP: This method is reliable for identifying and differentiating *E. histolytica, E. dispar,* and *E. moshkovskii* simultaneously from DNA samples taken from human feces. It is specific and sensitive, making it a valuable tool for improved clinical diagnosis during amoebic outbreaks and for managing endemic amoebiasis.

Multiplex PCR: Our multiplex PCR assay can identify *E. histolytica, E. dispar, E. moshkovskii, E. bangladeshi,* and *E. coli* in a single PCR reaction. It is a cost-effective and efficient technique that provides rapid and sensitive results for clinical diagnosis and epidemiological research, particularly for identifying *E. bangladeshi* in recent epidemiological studies.

TaqMan probe-based qPCR: We have developed highly specific and sensitive qPCR methods that are beneficial for diagnostic purposes and molecular epidemiological research related to *Entamoeba* spp.

These three new techniques offer reliable options for medical diagnosis and research, enabling a better understanding and management of amoebic infections.

CHAPTER X

SUMMARY

10.1. Summary

Diarrheal disease remains a significant health issue in both developed and developing nations, with parasites serving as prominent contributors to its prevalence. In our investigation, we conducted an active surveillance study to assess the prevalence of parasitic infections among individuals suffering from diarrhoea in the Kolkata metropolitan area and its vicinity. The findings from our research underscore the ongoing significance of parasites as causative agents of diarrheal illness within the studied region. Our observations revealed a substantial burden of *Giardia* and *Cryptosporidium* infections among the diarrheal patients. In our study on *Cryptosporidium* infections, *C. hominis* was found to be the predominant species, while we also observed a growing occurrence of *C. viatorum* as a newly emerging enteropathogen within our research area. It should be noted that *C. viatorum* was identified as a distinct species only in 2012, and it carries the risk of zoonotic transmission. Therefore, it is crucial to establish comprehensive control measures, precise diagnostic protocols, and effective treatment strategies to tackle this evolving public health issue.

During our investigation into amoebic infections, we made some significant discoveries. We found that *E. moshkovskii*, which was previously considered a harmless commensal, had the highest rate of occurrence among individuals with diarrhoea in our study. Our results suggest that *E. moshkovskii* is the primary cause of amoebic infections in the area we examined, followed by *E. histolytica* as the second most prevalent species. *E. dispar* cases were relatively uncommon in comparison. Moreover, we observed a notable prevalence of unidentified *Entamoeba* species, emphasizing the necessity for further investigations to enhance the identification and comprehensive characterization of these species within this geographical area. We hypothesized that *E. bangladeshi* might be a significant constituent of these unidentified *Entamoeba* species. We also conducted a population structure analysis of *E. moshkovskii*, revealing an expanding population of this species in our study area. Our research identified numerous clinically relevant SNPs and genotypes that may contribute to the pathogenicity of *E. moshkovskii*. Additionally, our investigation suggests that pigs could serve as a potential natural reservoir for *E. moshkovskii*, with the potential for zoonotic transmission of the pathogen. We strongly recommend the implementation of comprehensive planning, diagnosis, and treatment protocols for individuals infected with *E. moshkovskii* in eastern India. Furthermore, we encourage further studies to delve into the mechanisms of pathogenicity, aiming to identify the most promising drugs for controlling this pathogenic infection.

While *E. moshkovskii* is a common enteric parasite, *E. histolytica* is notably the most virulent enteric parasite species. To explore the genotypes linked to varying disease outcomes from *E. histolytica* infection, we conducted an MLST analysis using STR markers. Our findings revealed a distinctive genotype strongly associated with the formation of Amoebic Liver Abscess (ALA) caused by *E. histolytica*. Additionally, our study discovered several unique repeat patterns and genotypes not previously documented. This MLST analysis of *E. histolytica* is a valuable addition to worldwide genealogy research on this parasite.

It is noteworthy that the study has revealed a significant difference in the rate of cyst production between *E. histolytica* and other *Entamoeba* species. Moreover, several species of *Entamoeba* and *Cryptosporidium* exhibit identical morphology under a microscope. Therefore, microscopic examination alone is insufficient to distinguish them. Individual PCR or other techniques are also time-consuming and expensive. We have developed three distinct methods to identify and characterize common enteric parasites that are responsible for causing intestinal and extraintestinal disorders. These methods include Multiplex PCR, an 18S PCR RFLP technique, and a qPCR method. These methods also show promise for implementation in epidemiological studies. Choosing the appropriate technique to identify and study parasites is critical and depends on the intended purpose, available resources, and facilities.

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ORIGINAL ARTICLES

1. **Sardar SK**, Ghosal A, Haldar T, Das K, Saito-Nakano Y, Kobayashi S, Dutta S, Nozaki T, Ganguly S. Investigating Genetic Polymorphism in *E. histolytica* Isolates with Distinct Clinical Phenotypes. Parasitol Res. Accepted: Aug 18, 2023. https://doi.org/10.1007/s00436-023-07952-x

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RESEARCH

Investigating genetic polymorphism in *E. histolytica* isolates with distinct clinical phenotypes

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Abstract

Amoebiasis is an infection caused by enteric protozoa, most commonly *Entamoeba histolytica*, and is globally considered a potentially severe and life-threatening condition. To understand the impact of the parasite genome on disease outcomes, it is important to study the genomes of infecting strains in areas with high disease prevalence. These studies aim to establish correlations between parasite genotypes and the clinical presentation of amoebiasis. We employ a strain typing approach that utilizes multiple loci, including SREHP and three polymorphic non-coding loci (tRNA-linked array N-K2 and loci 1-2 and 5-6), for high-resolution analysis. Distinct clinical phenotype isolates underwent amplification and sequencing of studied loci. The nucleotide sequences were analysed using Tandem Repeats Finder to detect short tandem repeats (STRs). These patterns were combined to assign a genotype, and the correlation between clinical phenotypes and repetitive patterns was statistically evaluated. This study found significant polymorphism in the size and number of PCR fragments at SREHP and 5-6 locus, while the 1-2 locus and NK2 locus showed variations in PCR product sizes. Out of 41 genotypes, two (I6 and I41) were significantly associated with their respective disease outcomes and were found in multiple isolates. We observed that I6 was linked with a symptomatic outcome, with a statistically significant *p*-value of 0.0183. Additionally, we found that I41 was associated with ALA disease outcome, with a *p*-value of 0.0089. Our study revealed new repeat units not previously reported, unveiling the genetic composition of *E. histolytica* strains in India, associated with distinct disease manifestations.

Keywords Amoebiasis · *Entamoeba histolytica* · Diarrhoea · Amoebic liver abscess · STR

Introduction

Amoebiasis, which is caused by the microaerophilic/anaerobic protozoan parasite *Entamoeba histolytica*, is one of the most severe enteric infections in humans (Das et al. 2014). It is the third leading cause of death due to parasite infection after schistosomiasis and malaria (Singh and Galhotra 2014). The disease can spread via the faecal-oral route and is highly endemic in tropical and subtropical countries with an average socioeconomic status and poor public health (Guillén 2023; Preet et al. 2011). However, only one out of five individuals infected with *E. histolytica* develops intestinal or extraintestinal diseases, and the remaining 80% of infections are asymptomatic (Yanagawa et al. 2020). The specific determinants for this differential outcome of infection are yet to be fully understood, although host defence mechanisms and pathogen virulence are believed to be primary controlling factors. Some studies have reported that host immune responses, such as anti-inflammatory cytokines

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RESEARCH ARTICLE

Prevalence and molecular characterization of *Entamoeba moshkovskii* in diarrheal patients from Eastern India

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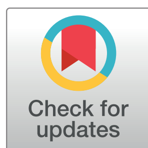

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Data Availability Statement: Representative sequences obtained in this study were deposited in GenBank under the accession numbers ON965383 - ON965450.

Abstract

Background

Importance of the amphizoic amoeba *Entamoeba moshkovskii* is increasing in the study of amoebiasis as a common human pathogen in some settings. Limited studies are found on the genetic and phylogenetic characterization of *E. moshkovskii* from India; hence remain largely unknown. In this study, we determined the prevalence and characterized the *E. moshkovskii* isolates in eastern India.

Methods

A three-year systemic surveillance study among a total of 6051 diarrhoeal patients from ID Hospital and BC Roy Hospital, Kolkata was conducted for *E. moshkovskii* detection via a nested PCR system targeting 18S rRNA locus. The outer primer set detected the genus *Entamoeba* and the inner primer pair identified the *E. moshkovskii* species. The 18S rRNA locus of the positive samples was sequenced. Genetic and phylogenetic structures were determined using DnaSP.v5 and MEGA-X. GraphPad Prism (v.8.4.2), CA, USA was used to analyze the statistical data.

Result

4.84% (95%CI = 0.0433–0.0541) samples were positive for *Entamoeba* spp and 3.12% (95%CI = 0.027–0.036) were infected with *E. moshkovskii. E. moshkovskii* infection was significantly associated with age groups (X^2 = 26.01, P<0.0001) but not with gender (Fisher's exact test = 0.2548, P<0.05). A unique seasonal pattern was found for *E. moshkovskii* infection. Additionally, 46.56% (95%CI = 0.396–0.537) were sole *E. moshkovskii* infections and significantly associated with diarrheal incidence (X^2 = 335.5,df = 9; P<0.0001). Sequencing

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RESEARCH

Development of a simple PCR–RFLP technique for detection and differentiation of *E. histolytica, E. dispar* and *E. moshkovskii*

Sanjib K. Sardar[1] · Seiki Kobayashi[2] · Koushik Das[1,3] · Yumiko Saito-Nakano[4] · Shanta Dutta[5] · Tomoyoshi Nozaki[6] · Sandipan Ganguly[1]

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Abstract
Epidemiological studies on amoebic infections are complicated by morphological overlap between the pathogenic *E. histolytica*, the commensal *E. dispar* and the amphizoic *E. moshkovskii*, necessitating molecular identification. The present study developed a simple and economical 18S PCR–RFLP method for the simultaneous detection and differentiation of the three species. PCR products were differentiated by Tat1 restriction digestion generating three different RFLP patterns. Validation was conducted by screening 382 faecal samples from human patients from Kolkata, India, hospitalized for diarrhoea. Analysis indicated that the PCR–RFLP could successfully differentiate between the three species and was confirmed by sequence analysis. This method could prove useful for clinical and epidemiological studies of amoebiasis.

Keywords Amoebiasis · *Entamoeba* spp. · PCR · PCR–RFLP · Restriction enzyme

Introduction

Amoebiasis, a disease caused by the enteric parasite *Entamoeba histolytica*, is common in low and lower-middle-income countries, where it is a leading cause of morbidity and mortality (Fischer Walker et al. 2012; Fontecha et al. 2015). It affects approximately 500 million people worldwide and is responsible for nearly 100,000 deaths annually, making it the third most important cause of parasite-associated mortality in humans after malaria and schistosomiasis (WHO, 1997; Solaymani-Mohammadi and Petri, 2008).

The prevalence of *E. histolytica* infection is often overestimated due to its morphologically similarity to other *Entamoeba* species, particularly the commensal *E. dispar* and the amphizoic *E. moshkovskii* (Soares et al. 2019). Recently, the PCR technique was accepted by the WHO as an alternative diagnostic tool for the detection of *E. histolytica* (Shirley et al. 2018). A wide variety of PCR techniques exist such as nested, multiplex and real-time PCR, and each has its advantages and limitations (Abe et al. 2002; Blessmann et al. 2002). Detection and differentiation of *E. histolytica/E. dispar/E. moshkovskii* by conventional/nested PCR for each species separately is highly sensitive, but a time-consuming procedure and expensive (Souza et al. 2007; Wang et al. 2014), and is also susceptible to contamination (Green and Sambrook, 2019). Multiplex PCR is a useful tool for the detection and differentiation of different *Entamoeba* species (Khairnar and Parija, 2007), but standardization of multiplex PCR can be difficult and non-specific amplification is also an issue (Fotedar et al. 2007). Multiplex PCR assays are not commercially available and test centres in endemic areas must standardize or develop their own protocols for the

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Research Article OPEN ACCESS

Molecular evidence suggests the occurrence of *Entamoeba moshkovskii* in pigs with zoonotic potential from eastern India

Sanjib K. Sardar[1], **Koushik Das**[1,2], **Maimoon Maruf**[1], **Tapas Haldar**[1], **Yumiko Saito-Nakano**[3], **Seiki Kobayashi**[4], **Shanta Dutta**[5] and **Sandipan Ganguly**[1,*]

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Abstract: *Entamoeba moshkovskii* Tshalaia, 1941 is prevalent in developing countries and it is considered to be primarily a free-living amoeba, which is morphologically indistinguishable, but biochemically and genetically different from the human infecting, pathogenic *Entamoeba histolytica* Schaudinn, 1903. The pathogenic potential of this organism is still under discussion. *Entamoeba moshkovskii* in human stool samples has been reported in different countries such as the United States, Italy, Australia, Iran, Turkey, Bangladesh, India (Pondicherry), Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil, but no data are available about the occurrence of *E. moshkovskii* in farm animals. This study provides data on the occurrence of *E. moshkovskii* in pigs in a total of 294 fresh faecal samples collected from five different regions in Kolkata, West Bengal, India. Stool samples were tested by nested PCR using primers targeting SSU rDNA of *E. moshkovskii.* The amplified PCR products were further confirmed by RFLP technique. Purified nested PCR products were also sequenced and identified via BLAST program run on the NCBI website to confirm species along with their genetic characteristics of the *E. moshkovskii* isolates. Overall 5.4% samples were identified as *E. moshkovskii* positive. Results of this study demonstrate that swine can host *E. moshkovskii* and should be considered as a potential natural reservoir for *E. moshkovskii*. However, the occurrence of *E. moshkovskii* infection in pigs was not statistically associated with their faecal consistency, sex and developmental stage.

Keywords: PCR, RFLP, host, natural reservoir, intestinal protists, diarrhea

The genus *Entamoeba* Casagrandi et Barbagallo, 1895 consists of at least nine species: *E. histolytica* Schaudinn, 1903, *E. dispar* Brumpt, 1925, *E. moshkovskii* Tshalaia, 1941, *E. bangladeshi* Royer, 2012, *E. coli* (Grassi, 1879), *E. nuttalli* (Catellani, 1908), *E. hartmanni* Prowazek, 1912, *E. polecki* Prowazek, 1912 and *E. chattoni* Levine, 1961, which are able to reside in the human intestinal lumen (Fotedar et al. 2007b, Delialioglu et al. 2008, Ngui et al. 2012). The first three species are the most prevalent and are morphologically similar under light microscope, but have different biochemical and genetic characteristics (Verweij et al. 2003). Although *E. polecki* has rarely been implicated as a cause of diarrheal disease, it is important to keep in mind that most of species are usually believed as commensal organisms of the human gut except *E. histolytica* and *E. moshkovskii* (see Salaki et al. 1979, Al-Areeqi et al. 2017). *Entamoeba histolytica* is considered as the most recognised pathogen of the human gut. Nonetheless, recent studies have reported the association of *E. moshkovskii* with gastrointestinal clinical manifestations indicating *E. moshkovskii* might be associated with pathogenicity (Ali et al. 2003, Fotedar et al 2007b, Khairnar and Parija 2007, Ngui et al. 2012). So far, the role of *E. moshkovskii* as an etiological agent of diarrhea in humans remains unclear (Clark and Diamond 1991).

Entamoeba moshkovskii was first described as a distinct species from Moscow by Tshalaia in 1941 (see Scaglia et al. 1983). It was primarily considered to be a free-living environmental strain *Entamoeba* sp. and is still regarded as a common protist species found in anoxic sediments and brackish coastal pools. It is osmotolerant in nature and can be cultured in various media suitable for intestinal protists, in which it grows easily at temperatures of 10–15 °C and 37 °C (Diamond and Bartgis 1970, Scaglia et al. 1983, Clark and Diamond 1991).

In 1961, an *E. histolytica*-like strain was obtained from a resident of Laredo, Texas, who suffered from diarrhea, weight loss and epigastric pain; this strain was named as

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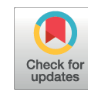

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BRIEF COMMUNICATION

Molecular Identification of *Cryptosporidium viatorum* Infection in a Patient Suffering from Unusual Cryptosporidiosis in West Bengal, India

Sanjib Kumar Sardar[1], Ajanta Ghosal[1], Yumiko Saito-Nakano[2], Shanta Dutta[3], Tomoyoshi Nozaki[4], Sandipan Ganguly[1,*]

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Abstract: In this study, we have collected and screened a total of 268 stool samples from diarrheal patients admitted to an Infectious disease hospital in Kolkata for the presence of *Cryptosporidium* spp. The initial diagnosis was carried out by microscopy followed by genus specific polymerase chain reaction assays based on 70 kDa heat shock proteins (HSP70). DNA sequencing of the amplified locus has been employed for determination of genetic diversity of the local isolates. Out of 268 collected samples, 12 (4.48%) were positive for *Cryptosporidium* spp. Sequences analysis of 70 kDa heat shock proteins locus in 12 *Cryptosporidium* local isolates revealed that 2.24% and 1.86% of samples were showing 99% to 100% identity with *C. parvum* and *C. hominis*. Along with the other 2 major species one recently described globally distributed pathogenic species *Cryptosporidium viatorum* has been identified. The HSP70 locus sequence of the isolate showed 100% similarity with a previously described isolate of *C. viatorum* (Accession No. JX978274.1, JX978273.1, and JN846706.1) present in GenBank.

Key words: *Cryptosporidium viatorum*, cryptosporidiosis, molecular characterization, 70 kDa heat shock proteins (HSP70).

Cryptosporidium spp. are leading cause of diarrhoea and malnutrition, particularly in the developing countries around the world. At least 34 different species and more than 40 genotypes of *Cryptosporidium* have been described to infect humans and other animals and numerous of which are reported to have zoonotic potential [1,2]. The majority of the infections caused by the 5 species of *Cryptosporidium* are- *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium felis,* and *Cryptosporidium canis*, of which the first 2 are the major causative agent of cryptosporidiosis in human [3,4]. *Cryptosporidium viatorum* was first described in 2012 from travellers returning to Great Britain from India, Nepal, Bangladesh and Pakistan [5-7].

The acute symptoms associated with cryptosporidiosis due to *C. viatorum* infection in the travellers returning to Sweden and Great British from Bangladesh, India, Nepal, Pakistan Kenya, and Guatemala have included diarrhoea, abdominal pain, headache, vomiting nausea, fever and marked weight loss with illness lasting from 9 to 30 days [6,8]. Although it has been observed that infection with more uncommon species/genotypes is not inevitably associated with the immune status of the hosts[9]. *Cryptosporidium* can be diagnosed by a number of techniques including of identification of oocysts in stools using modified acid fast staining but this technique does not allow to identify the species as the oocysts are morphologically indistinguishable and measure 5.35×4.72 µm with a length to width ratio of 1.14 [5]. For prevention and control of cryptosporidiosis, identification and characterization of different *Cryptosporidium* species along with determination of population variants (genotypes and subtypes) are fundamental in the study of cryptosporidiosis epidemiology. As the oocysts of many species of *Cryptosporidium* are indistinguishable from each other, molecular techniques are necessary for identification of species and determination of genotype and subtype in order to specify the organism responsible for infection and the source and routes of transmission.

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Case Report

First case report of Cyclosporiasis from eastern India: Incidence of *Cyclospora cayetanensis* in a patient with unusual diarrheal symptoms

Sanjib K Sardar[1 #], Gaurav Goel[2 #], Ajanta Ghosal[1], Rasika Deshmukh[2], Sanjay Bhattacharya[2], Tapas Haldar[1], Md. Maimoon Maruf[1], Rajkumar Mahto[2], Jeevan Kumar[3], Saurabh J Bhave[3], Shanta Dutta[4], Sandipan Ganguly[1]

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Abstract

Cyclospora cayetanensis, a recently described coccidian parasite causes severe gastroenteric disease worldwide. Limited studies are found on the incidence of *C. cayetanensis* infection from India; hence remains largely unknown. To date, no case of cyclosporiasis from eastern India has been reported. In this study, we described an incidental case of *C. cayetanensis* in a 30 years old Bengali female patient with no travel history from eastern India. In June 2022, the patient presented with a history of diarrhoea persisting for more than two months with continuous passage foul smelling stools for which she took multiple antibiotics that were ineffective. There were no Salmonella, Shigella, or Vibrio-like organisms in the patient's faecal sample, and Toxin A/B of Clostridium difficile was also not detected by ELISA. The patient was HIV-negative. Finally, UV autofluorescence and DNA-based diagnosis confirmed the presence of *C. cayetanensis*, and the treatment with a combination of appropriate antibiotics was successful. This case report could raise awareness about *C. cayetanensis* associated diarrhoeal cases in India.

Key words: Cyclosporiasis; *Cyclospora cayetanensis;* diarrhoea; India.

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Background

Cyclospora cayetanensis is a recently described intestinal protozoan parasite belonging to the family *Eimeriidae*, subclass Coccidia, phylum Apicomplexa [1,2]. It was first reported to be an unidentified Isospora-like coccidian parasite causing diarrhoea in patients of Papua New Guinea by Ashford (1979) [2]. Later, in 1994, Ortega, Gilman & Sterling proposed the name *C. cayetanensis* after observing the sporulation and excystation of the organism and the unique morphological features of its oocysts via both light and electron microscopy [2]. Until now, *C. cayetanensis* has been identified solely from the human enteric tract and is now considered an emerging organism, able to cause a severe gastro-enteric disease called cyclosporiasis, especially in immunocompromised individuals, children, and the elderly [2]. Human cyclosporiasis can range from asymptomatic to severe, and if not treated, clinical symptoms can persist for several weeks to a month or more [3,4]. The clinical outcomes are associated with the age and immune response of the host [2,4]. Presently, the biology, risk factors, and routes of transmission of *C. cayetanensis* remain poorly understood [5]. *C. cayetanensis* transmitted to new human hosts via the faecal-oral route or through food and water contaminated by oocysts [6]. Non-sporulated oocysts of *C. cayetanensis* require a maturation period of 7–14 days outside the host body under favourable environmental conditions and thus become infectious [7]. The infective oocysts contain two ovoid sporocysts, each containing two sporozoites. Therefore, the oocysts in fresh stool are non-infectious and it is highly unlikely to be infected by this parasite via fresh stool through faecal-oral contact [8].

Cyclospora is responsible for several outbreaks worldwide in the last two decades, despite having endemicity in only tropical and sub-tropical areas [8]. Although, most of the cases reported in non-endemic

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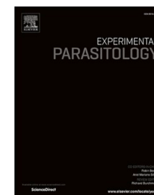

Functional characterization of phospholipase B enzyme from *Giardia lamblia*

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ABSTRACT

The microaerotolarent amitochondriate protozoan *Giardia lamblia* causes Giardiasis and produces a unique enzyme called Phospholipase B (PLB) in contrast to higher eukaryotes. The enzyme is produced upon induction with oxidative (H2O2) stress, thus leading to prostaglandin E2 (PGE2) production. It exists in dimeric form, and its molecular weight is 56 kDa. This PLB was extracellularly cloned in the pET21d vector. The ORF is 1620 bp (Genbank accession no. -OM939681) long and codes for a protein 539 amino acid long, with a 15 amino acid long amino-terminal signal peptide. The highest enzyme activity of PLB was identified at pH 7.5 and 35 °C. This specific enzyme was also active at 50 °C pH 10, but activity was low. We also analyzed the expression of PLB protein in *G. lamblia,* which was significantly induced under *increased* oxidative stress.

1. Introduction

Early-branched amitochondriate protozoa, *Giardia lamblia*, causes diarrheal diseases in humans and animals. Approximately 280 million cases of Giardiasis are reported globally (Lane and Lloyd, 2002).*Giardia* infection can be asymptomatic or symptomatic; symptoms include diarrhoea, abdominal discomfort, vomiting, malabsorption, and weight loss (Kamda and Singer, 2009). The life cycle of this intestinal parasite consists of two morphological forms: trophozoites and cysts. The trophozoite form colonizes in the small intestine, whereas the resistant cysts are responsible for the transmission of the disease through contaminated food and water. Previous epidemiological studies showed that humans, cattle, and other mammals can be the hosts for this parasite and chronic infection has a negative impact on the global economy (Giangaspero et al., 2005). Available data supports the zoonotic transmission of this parasite among different host species through contaminated water (Monis and Thompson, 2003; Smith et al., 2007; Bajer, 2008). After entry to the small intestine, two binucleated trophozoites are emerged from the cyst and localize in the duodenum. Inside the host gut environment, the trophozoites are exposed to complex and ever-changing hydrogen ion concentrations and comparatively high oxygen levels (60 μM) (Davenport, 1977; Atkinson and Zuckerman, 1980).

Reactive oxygen species (ROS) production is an inevitable consequence of normal oxidative metabolism. Low levels of ROS can play a role in cell signalling by acting as direct cellular stimuli or as receptor-directed stimuli (Balboa and Balsinde, 2006). When in high levels ROS exerts many direct and indirect effects on cell signalling pathways, which finally result in the induction of apoptosis or necrosis (England and Cotter, 2005). One of the effects of ROS on cells is lipoperoxidation, i.e., the oxidative modification of membrane phospholipids. Lipid peroxidation predominantly occurs at the sn-2 position of phospholipids, as most of the unsaturated and polyunsaturated fatty acids are esterified in this specific position. This position is also targeted by phospholipase A2 (PLA2) for cleavage (Cummings et al., 2000). Oxidative stress and peroxidation of membrane phospholipids are positively correlated with the enhanced PLA2 activity in several organisms (Sapirstein and Bonventre, 2000). Increased PLA2 activity in cells under oxidative stress can generate several biologically active mediators, such as arachidonic acid (AA) and associated metabolites (Balboa and Balsinde, 2006; Lister

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Parasitology Research
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1 RESEARCH

2 Genotyping and epidemiological distribution of diarrhea-causing 3 isolates of *Giardia duodenalis* in southeastern part of West Bengal, 4 India

5 Ajanta Ghosal[1] · Sanjib K. Sardar[1] · Tapas Haldar[1] · Maimoon Maruf[1] · Yumiko Saito-Nakano[2] · Shanta Dutta[3] ·
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AQ1 **Abstract**

10 The prevalence and genetic diversity of the protozoan pathogen *Giardia duodenalis* have been extensively studied worldwide.
11 There is currently a lack of data regarding the genetic variability of the organism in eastern India. Understanding the circulating
12 genotypes and associated risk factors is crucial for effective planning and implementing control measures. Therefore, the objec-
13 tive of the study was to conduct an epidemiological study to determine the prevalence and identify the various genotypes present.
14 This survey adds to our knowledge on the occurrence and distribution of *Giardia* genotypes in the studied region. The overall
15 prevalence was found to be 6.8%. This parasitic infection was significantly associated with two age groups, i.e., >0–5 years and
16 >5–12 years. Using a multilocus genotyping method, we genotyped 52 human *Giardia* isolates that were obtained from diar-
17 rheal patients. Two distinct assemblages were found in the population—30.8% belonged to assemblage A; 63.5% belonged to
18 assemblage B, prevalent in the population; and 5.7% belonged to a combined assemblage A+B. Sub-assemblage AII was found
19 in 17.3% of the cases, followed by sub-assemblage AI (13.5%). High levels of genetic diversity were found within the popula-
20 tion of assemblage B undergoing balancing selection. Overall, the high prevalence of the parasite observed, particularly among
21 children, raises a major concern and necessitates implementation of robust control measures. Furthermore, we report the presence
22 of numerous unique genotypes, circulating in this limited geographical boundary, which can be useful dataset for future studies.

23 **Keywords** *Giardia* · MLG · SNP · Balancing selection · West Bengal · India

AQ2 Introduction

25 Enteric parasites contribute significantly to the burden of
26 diarrheal diseases worldwide. While parasitic diseases run
27 rife in developing countries, sporadic cases and outbreaks
28 are also reported in developed countries (Fletcher et al.
29 2012). *Giardia duodenalis* (formerly known as *G. lamblia* or AQ3
30 *G. intestinalis*) is a non-invasive intestinal protozoan which
31 is commonly associated with diarrheal illnesses (namely
32 giardiasis) in humans, livestock, and wild animals (Takumi
33 et al. 2012). About 280 million cases are reported annually
34 (Lane and Lloyd 2002; Squire and Ryan 2017), with more
35 than 2.5 million annual diarrhea cases coming from devel-
36 oping countries (Thompson 2000). Infection is initiated in
37 the host when the ingested cysts excyst into trophozoites in
38 the duodenum (proximal part of the small intestine) after
39 passing through the acidic environment of the stomach. The
40 trophozoites replicate and remain adhered on the mucosal
41 surface of the intestine, where it causes malabsorption
42 resulting in diarrheal symptoms. Exposure to biliary fluid
43 causes trophozoites to turn into the resistant "cyst" form in
44 the jejunum, which are then shed with the feces to the envi-
45 ronment, thereby completing the transmission cycle via the
46 fecal-oral route (Adam 2001).

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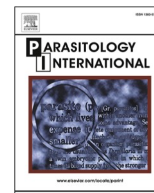

Multilocus sequence typing (MLST) of *Entamoeba histolytica* identifies *kerp2* as a genetic marker associated with disease outcomes

Koushik Das [a,1], Sanjib kumar Sardar [a], Ajanta Ghosal [a], Yumiko Saito-Nakano [b], Shanta Dutta [a], Tomoyoshi Nozaki [c], Sandipan Ganguly [a,*]

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A R T I C L E I N F O

Keywords:
Entamoeba histolytica
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Disease outcome
Amoebiasis
Population genetics

A B S T R A C T

Amoebiasis caused by protozoan parasite *Entamoeba histolytica* has diverse infection outcomes. The relationship between parasite genotypes and outcome of amoebic infection is still a paradox and needs to be explored. Genome information of infecting strains from endemic areas throughout the world is essential to explore this relation. Comparative genetics between *E. histolytica* populations from different disease outcomes have been studied to identify potential genetic markers having single nucleotide polymorphisms (SNPs) significantly associated with specific clinical outcome. Coding and non-coding regions have significantly different rates of polymorphism. Non-synonymous base substitutions were significantly more frequent than synonymous within coding loci. Both synonymous and non-synonymous SNPs within lysine- and glutamic acid rich protein 2 (*kerp2*) locus were significantly associated with disease outcomes. An incomplete linkage disequilibrium (LD) value with potential recombination events and significant population differentiation (F_{ST}) value have also been identified at *kerp2* locus within the study population. Presence of disease specific SNPs, potential recombination events, and significant F_{ST} value at *kerp2* locus indicate that *kerp2* gene and its gene product are under constant selection pressure exerted by host on parasite and could also be a potential determinant of disease outcome of *E. histolytica* infection. Furthermore, *E. histolytica* isolated from asymptomatic carriers are phylogenetically closer to those causing liver abscess in human and exhibit potential inter-population recombination among them. Individuals with persistent asymptomatic *E. histolytica* infection may be under high risk of developing amoebic liver abscess formation in future and detailed investigation of asymptomatic individuals from endemic areas should be always required.

1. Introduction

Amoebiasis caused by protozoan parasite *Entamoeba histolytica* is one of the major enteric diseases in human. It, being one the foremost parasitic disease after malaria is responsible for approximately 100,000 human deaths per annum [1]. 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) [2,3]. Specific determinants for these diverse disease outcomes still remain elusive; however, host genetics and parasite genotypes could be two possible factors [4,5]. Genome information of infecting strains from endemic areas throughout the world is certainly crucial to determine the exact genetic traits of parasite affecting its virulence capacity as well as different disease causing abilities. Selection of suitable genetic markers is needed for an optimum genotyping system. Since *E. histolytica* does not appear to contain microsatellite, measurement of genetic diversity and estimation of population structure has relied upon other polymorphic repetitive markers like serine-rich *E. histolytica* protein (*SREHP*) gene, chitinase (*CHI*) gene, and tRNA-linked STR loci [6]. However, genotyping studies based upon variations in these repetitive DNA often indicate very high levels of genetic diversity in an *E. histolytica* population [7–12]. This also results in the identification of immense variety of novel genotypes from different parts of the world [13–16], which are

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EC ENDOCRINOLOGY AND METABOLIC RESEARCH

Review Article

Soil-transmitted Helminths Infection in India

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Abstract

Soil-transmitted Helminths (STHs) form one of the most significant groups of infectious agents that cause major public health problem in tropical and sub-tropical countries. The roundworm (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*) and hookworms (*Ancylostoma duodenale* and *Necator americanus*) are the major species that infect people and causes ascariasis, hookworm disease and trichuriasis respectively. According to WHO STHs is regarded as a neglected tropical disease.

Keywords: *Soil-Transmitted Helminths (STHs); Ascariasis; Hookworm; Trichuris trichiura; WHO; Tropical*

Introduction

Soil-transmitted helminths (STHs) form one of the most significant groups of infectious agents that are cause of a major public health problem in tropical and sub-tropical countries [1]. It infects nearly 2 billion people of world's population of which children are mostly affected [2,3]. According to the World Health Organization (WHO) it is estimated that more than 870 million children live in the endemic areas [4].

Disease burden

Globally the most significant STHs are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* or *Ancylostoma duodenale*). According to the latest data the estimated number of people infected with roundworms is 807 million, whipworms is 604 million and hookworm is 576 million [5,6]. Sub-Saharan Africa (SSA), East Asia, China, India and South America are the most affected countries with this commonly neglected tropical disease [3]. Nearly 25% to the total global cases with 220.6 million children need preventive chemotherapy in India [7].

Disease transmission

The most vulnerable group of people affected by the disease are school going children. Water quality, sanitation, hygiene practices and socio-economic status in the affected areas are closely associated with the prevalence of STH infection [5]. It is regarded as one of the world's most important causes of malnutrition, poor school performance, delayed physical growth and impaired cognitive function [6].

Aim and treatment

Aim of the World Health Organization (WHO) is to control of STH in children to reduce morbidity by 2020 through conducting the deworming program in schools [8]. The WHO recommends biannual treatments in areas with over 50% prevalence of STH and annual

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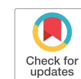

Genetic characterization reveals evidence for an association between water contamination and zoonotic transmission of a *Cryptosporidium* sp. from dairy cattle in West Bengal, India

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A B S T R A C T

Cryptosporidium sp. is an enteric parasite with zoonotic potential, and can infect a wide range of vertebrates, including human. Determining the source of infection and the mode of transmission in a new endemic region is crucial for the control of cryptosporidiosis. In the present study, we have assessed the importance of dairy cattle as a potential source of *Cryptosporidium* infection for humans in a newly recognized endemic region. *Cryptosporidium* isolates from dairy calves, humans (farm workers) and nearby water bodies were genetically characterized based on *18SrRNA* and *hsp70* genes. A high incidence of *Cryptosporidium* infection was identified in our study region. This finding is of public health concern. *Cryptosporidium ryanae* rather than *Cryptosporidium parvum* has been identified as the most prevalent infecting species in the study region. Infections were associated with clinical symptoms of infected animals. An incomplete linkage disequilibrium (LD) value with potential recombination events at *18SrRNA* locus were identified for the first time in *C. ryanae*, which was previously reported as a clonal population. Phylogenetic analysis revealed the presence of identical genotypes of a *Cryptosporidium* sp. from dairy calves, farm workers and nearby water bodies and indicates an association between water contamination and zoonotic transmission of Cryptosporidiosis in our study region.
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1. Introduction

Cryptosporidium is a coccidian parasite infecting a wide range of vertebrates, including humans. Disease transmission typically occurs via fecal-oral route following either direct contact with contaminated fecal samples from an infected host or indirectly through contaminated water or food. Cattle is a major reservoir for *Cryptosporidium* spp. (Zhao et al., 2014). Individuals in close proximity with infected animals are at a high risk of acquiring cryptosporidiosis, especially cattle handlers, veterinarians and others working in low hygiene areas. The disease is usually self-limiting in immunocompetent individuals, but can be life-threatening or fatal among malnourished and immunocompromised patients (Steeb et al., 1987; Kurniawan et al., 2013). Unlike

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Archives of Parasitology

Ganguly et al., Arch Parasitol 2017, 1:3

Case Report Open Access

Intractable Seizure in a Case of Primary Amoebic Meningoencephalitis caused by the Free Living Amoeba *Naegleria Fowleri*

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Keywords: Meningoencephalitis; Intractable seizure; Coma; Amoeba; *Naegleria fowleri*

Case Report

Naegleria fowleri is only one among the many species of the genus *Naegleria* composed of a group of free living amoeboflagelates and this particular species can cause human disease if infected. The disease, namely Primary Amoebic Meningoencephalitis (PAM or PAME) caused by this amoeboid excavates *Naegleria fowleri*, is a very rare but fatal disease of the central nervous system. The disease is not very common, however, these days its traces are found in various countries around the globe. Sometime it becomes fatal, taking toll of human lives. Quite surprisingly, the presence of *N. fowleri* has been reported in various kinds of water bodies. It may be domestic water bodies, polluted water bodies from industrial waste as well as water from the swimming pools, water sport and amusement parks. The presence of *N. fowleri* in different water bodies is associated with various factors like temperature, pH, presence of coliforms and the amount of organic matter present. Water rich in iron and iron containing compound favour the growth of *N. fowleri*. As the cases of PAM are showing up these years, it is becoming a matter of utmost concern [1].

Here, we report a case of Primary Amoebic Meningoencephalitis in a 15 year old, male patient, who was admitted at Kothari Medical Centre, Kolkata, India in early summer of April 2015. He was admitted with a history of fever, headache, altered sensorium and intractable seizures for 5-7 days at the time of admission.

The patient was unconscious when admitted (GCS of E_1 V_1 M_5). His pulse rate was regular at 98/min, blood pressure was 170/100 mmHg and the patient had fever (102°F). Neurological examination revealed neck rigidity with bilateral planter extensor reflex. Blood tests were performed to diagnose the infection causing agent. Widal test, HIV I and II, Dengue serum IgM and IgG test and test for malaria parasite were found to be negative. Blood test showed haemoglobin levels at 11.6 g/dL and WBC counts at 7400 cells/μL. Serum electrolytes, sodium and potassium were found to be 144 mEq/L and 3.8 mEq/L respectively. His relatives informed that he used to take bath in dirty river water.

On radiological imaging (CT scan and MRI of brain) no abnormality was detected.

Examination of CSF showed a total cell count of 210 cells/cumm (polymorph 12%, mono nuclear cells 88% with plenty RBC), protein 200 mg/mL, sugar 64 mg/dL. Random corresponding blood sugar was 84 mg/dl. He was put on ventilator support.

CSF was collected from the patient and used for microscopy and DNA isolation using Qiagen Blood DNA kit. CSF samples were observed for trophozoite or cyst [1,2]. Wet mount preparation of the CSF showed pear shaped motile trophozoites form of organism with actively directional movement suggesting free living amoeba. The cytoplasm was granular in nature and central nuclear karyosome was seen. Aerobic culture of the CSF did not show any growth. No AFB was seen in Z-N stain. India ink preparation did not reveal any capsulated organism suggestive of fungal infection. Phase Contrast microscopy showed typical morphology of *Naegleria spp*. PCR was performed by amplifying the *Naegleria* specific gene *Mp2Cl5* as described earlier [1]. By performing the nested PCR, typical *Naegleria* specific band of 110 bp was observed in 1.5% agarose gel electrophoresis (Figures 1 and 2) [1].

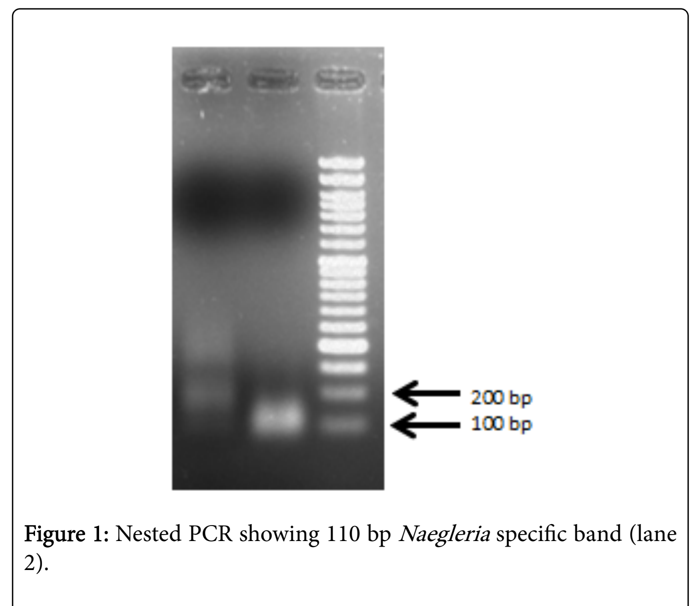

Figure 1: Nested PCR showing 110 bp *Naegleria* specific band (lane 2).

The disease is very serious and often fatal [2]. The patient unfortunately died 10 days after admission. The disease was first

Excystation and phylogenetic analyses of *Chilomastix* and *Retortamonas* species

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Manuscripts

Under Review

Jun Suzuki; Sanjib Kumar Sardar; Ajanta Ghosal; Naoko Yoshida; Hanako Kurai; Yudai Alex Takahashi; Yumiko Saito-Nakano; Sandipan Ganguly; Seiki Koayashi

Excystation and phylogenetic analyses of *Chilomastix* and *Retortamonas* species

Excistação e análises filogenéticas das espécies *Chilomastix* e *Retortamonas*

Abstract

In vitro excystation of suspected *Chilomastix mesnili* and *Retortamonas* sp. from *Urba auropuncta* and *Macaca fusucata* was induced using an established protocol for *Giardia intestinalis* modified with H_2S-rich Robinson's medium supplemented with *Desulfovibrio desulfuricans*. Phylogenetic analysis of excysted flagellates based on their 18S rRNA genes revealed that *C. mesnili* from a Japanese macaque (*M. fusucata*) belonged to the same cluster as *C. mesnili* isolates from humans [sequence identity to KC960589 (88.3%) and KC960590 (88.5%)], whereas the mammalian isolate of *Retortamonas* spp. from a small Indian mongoose (*U. auropuncta*), which habitually feeds on frogs, belonged to the same cluster as that of the amphibian isolate of *Retortamonas* spp. from a "poison arrow frog" [sequence identity to AF439347 (94.9%)].

Keywords: excystation, *Chilomastix mesnili*, *Retortamonas* spp., *Macaca fusucata*, *Urba auropunctata*, phylogenetic analysis

Resumo

A excistação in vitro das espécies suspeitas *Chilomastix mesnili* e *Retortamonas*, retiradas a partir da *Urba auropuncta* e da *Macaca fusucata*, foi induzida usando um

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Giardia, giardiasis and its zoonotic transmission

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Abstract

Giardia lamblia is the most common gastrointestinal disease causing agent in developed and developing countries. It has been reported that more than 280 million people are affected by acute and chronic giardiasis annually worldwide. The outcomes of *G. lamblia* infections are highly variable because of the mixed infections with other microorganisms. Majority of infected individuals are below 5 years. They are usually suffering from stomach cramp, irritable bowel syndrome, nausea and vomiting. Specific determinants for these diverse disease outcomes still remain elusive. Proper identification and genetic characterization of clinical isolates from endemic areas throughout the world becomes a certainly efficient tool to determine the potential genetic traits of parasite affecting its virulence capacity as well as different disease causing abilities. From the previous molecular biology studies, in both humans and animals, it is clear that predominance of the zoonotic Assemblage E subtype family of *Giardia* highlights the likely occurrence of zoonotic transmission in Indian cattle of giardiasis in West Bengal, Kolkata.

This communication explores the fact that gastrointestinal diseases are the most numerous in humans because *Giardia* cyst is constantly exposed to the environment. The chapter throws light on protozoan diseases and highlights that low personal hygiene and contacts with animals are important predictors for intestinal protozoan infections. As it will emerge out from this communication, both anthroponotic and zoonotic transmissions play potential roles in the transmission of giardiasis in the community. It would also be clear as to how the molecular genotyping may assist in developing effective control strategies based on a better understanding of epidemiology of this parasite.

Keywords

Giardia lamblia, Giardiasis, zoonosis, India, protozoal infections

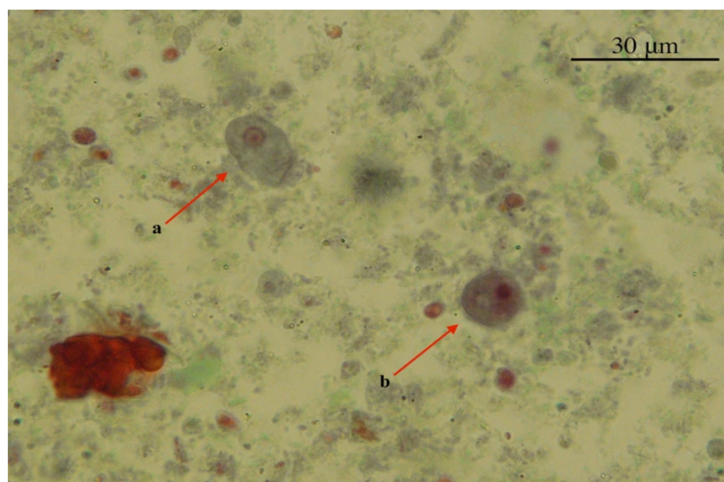
Introduction

Giardiasis, the most common gastrointestinal parasitic disease that is characterized by acute or chronic diarrhoea, is caused by protozoan parasites *Giardia* (Bhattacharya et al. 2002). It is the major anaerobe and resides in mammalian gut. *Giardia lamblia* is the only species known to us

RESEARCH HIGHLIGHT | 24 June 2023

A new diarrhoea bug infects people in east India

82 years after it was first reported in Moscow, the seasonal pathogen infecting more children than adults, surfaces in samples from Kolkata



Microscopic view of *Entamoeba moshovskii* trophozoites (a) and cysts (b). Credit: ICMR-NICED, Kolkata

A three-year surveillance study reveals the presence of a previously unreported amoeba pathogen in children and adults in Kolkata[1]. The pathogen, known as *Entamoeba moshkovskii*, triggers amoebiasis, a disease that affects the intestines.

Researchers identified a genetic marker in this pathogen that could potentially be used to distinguish it from other Entamoeba species in clinical samples.

Since its first diagnosis in Moscow in 1941, the parasite has been detected in other countries. But, India lacks data on this parasite. To determine its prevalence in India, the scientists collected more than 6000 clinical samples from two Kolkata-based hospitals and screened them using microscopic and molecular tools.

The team, which included researchers at the ICMR-National Institute of Cholera and Enteric Diseases in Kolkata, found that 3.12% of the samples were infected with *E. moshkovskii*. The number of cysts, the resistant and transmittable form of the parasite, was smaller than other Entamoeba species. Of the 189 positive samples, only 9% showed the presence of trophozoites, the motile form of the parasite. The bug's infection rate was highest among children between 5 and 12 years, and the lowest among adults between 19 and 29 years. The parasite's prevalence was higher than that of *Entamoeba histolytica* and it peaked during the post-fall and summer season.

A few changes in single DNA bases in a specific gene might have increased the fitness of the parasite, allowing it to adapt to the gut environment, the researchers note.

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A new diarrhoea-causing parasite found

The Hindu Bureau

A three-year surveillance study from March 2017 to February 2020 in Kolkata has found an amoeba pathogen that previously did not cause any amoebiasis (a form of diarrhoea) in humans has now become pathogenic. Surprisingly, a team of researchers from the Kolkata-based National Institute of Cholera and Enteric Diseases (ICMR-NICED) found that not only had the amoeba pathogen – *Entamoeba moshkovskii* – turned pathogenic, it was the leading cause of amoebic infections in humans; more than half of the amoebic infections were caused by this. The researchers studied the stool samples of diarrheal patients in Kolkata. Of particular concern is the fact that infections caused by *E. histolytica*, which used to be the predominant amoeba pathogen that caused amoebiasis, were decreasing and the newly pathogenic *E. moshkovskii* was taking its place. The researchers identified a few mutations that signify an essential role of the new pathogenic parasite in adapting to the gut environment of humans or in acquiring other enteric pathogens.

The results of the study were published recently in the journal *PLOS Neglected Tropical Diseases.*

Diarrhoea can be caused by bacteria, viruses and amoeba pathogens. In the study, the team of researchers led by Dr. Sandipan Ganguly of NICED found nearly 5% of patients with diarrhoea that was caused by different *Entamoeba* species and over 3% of patients were infected with *E. moshkovskii.* While there were no statistically significant differences between infections in males and females, the infections were most predominant in children aged 5-12 years.

While infections caused by *E. histolytica* peaked during the wet season and decreased with the arrival of the dry season, the seasonal pattern of *E. moshkovskii* infection in Kolkata was quite unique – there were two infection peaks coinciding with summer and post-fall season. During the two-decade-long active surveillance study for the detection of common enteric parasites in and around Kolkata, the researchers found infections coming up during non-seasonal periods for *E. histolytica*. They also observed a significant proportion of cysts/trophozoites of amoeba in stool samples that have a similar morphological feature to *E. histolytica*.

Predominant: More than half of amoebic infections in Kolkata were caused by *Entamoeba moshkovskii*

When they carried out PCR-based molecular identification to identify the similar looking amoeba trophozoites, they found that the morphologically indistinguishable amoeba from *E. histolytica* was indeed the related species *E. moshkovskii*.

According to the authors, another notable feature was that infection with the new pathogenic amoeba alone was statistically associated with diarrhoeal occurrence. "The diarrheal incidents associated with *E. moshkovskii* were not commonly coinfected in Kolkata. It acts as a "potential" pathogen causing diarrhoea and other gastrointestinal disorders in the study area," they write.

Amoebiasis is routinely diagnosed by light microscopy. But light microscopy has limited sensitivity and specificity, hence it becomes difficult to differentiate between the cyst and trophozoites of the pathogenic *E. histolytica* and *E. moshkovskii*. While trophozoites of *E. histolytica* are generally found in large numbers in stool samples, that is not the case with *E. moshkovskii*.

So in order to identify the similar looking trophozoites and identify the pathogenic amoeba that was causing diarrhoea during off-seasons, the researchers turned to PCR-based molecular identification. This led to the identification of *E. moshkovskii* in over 50% of diarrhoea cases caused by amoebic parasites.

The team has so far not carried out drug-susceptibility tests for *E. moshkovskii*.