Detection of Common Enteric Parasites in Kolkata and Characterisation of the Pathogenic Factors of Local Isolates of *Giardia lamblia*

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled " **DETECTION OF COMMON ENTERIC PARASITES IN KOLKATA AND CHARACTERIZATION OF THE PATHOGENIC FACTORS OF LOCAL ISOLATES OF *Giardia lamblia*"** submitted by Smt. **Ajanta Ghosal** who got her name registered on **18th September, 2018** for the award of Ph.D. (Science) Degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Sandipan Ganguly,** Scientist F and Head, Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, India and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

5 Sep 2024

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PREFACE

This Ph.D. thesis was prepared at the **Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases,** Kolkata, India to fulfil the requirements for obtaining a Ph.D. degree. The thesis is titled "**DETECTION OF COMMON ENTERIC PARASITES IN KOLKATA AND CHARACTERIZATION OF THE PATHOGENIC FACTORS OF LOCAL ISOLATES OF *Giardia lamblia***" and the research was conducted at the **Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases,** Kolkata, India. The thesis was completed under the direct guidance of **Dr. Sandipan Ganguly**, 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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Date: 05.09.24

Place: Kolkata

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This thesis is dedicated to my loving parents, my teachers and friends for their unwavering support and encouragement throughout the journey.

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A Ph.D. is not only an academic achievement, it's a transformative journey that moulds and defines who we become. It teaches us resilience, determination, and the importance of pursuing our dreams fearlessly. It is a dream, no matter how challenging, for in the end, it's not just about the destination but the person you become along the way. In this challenging yet inspiring journey many individuals have played important role and I would like to gladly acknowledge them.

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Date: 05.09.24

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CONTENTS

ABBREVIATION

ORT	Oral rehydration therapy
DALY	Disability-adjusted life year
WHO	World Health Organization
FAO	Food and Agricultural Organization of the United States
Mb	Megabase pair
sRNAs	Small Ribonucleic Acids
tRNA	Transfer Ribonucleic Acids
endo-siRNA	Endogenous small interfering RNA
miRNA	Micro Ribonucleic Acids
gdh	Glutamate dehydrogenase
bg	Beta giardin
tpi	Triosephosphate isomerase
SSU rRNA	Small Subunit Ribosomal Ribonucleic Acid
VSP	Variable surface protein
MLG	Multilocus genotypes
NTDI	Neglected Tropical Disease Initiative
PVA	Polyvinyl alcohol
SAF	Sodium acetate-acetic acid-formalin
MIF	Merthiolate-iodine-formalin
IFA	Immunofluorescence assay
PCR	Polymerase Chain Reaction
SSU rDNA	Small Subunit Ribosomal Deoxyribonucleic Acid
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
ELISA	Enzyme-linked immunosorbent assay
ef-1α	Elongation factor 1-alpha
GLORF-C4	*Giardia lamblia* open reading frame C4
IGS	Intergenomic rDNA spacer region
ASH	Allelic sequence heterogeneity
DNA	Deoxyribonucleic acid
PFOR	Pyruvate-ferredoxin oxidoreductase
TrxR	Thioredoxin reductase
FDA	Food and Drug Administration
MTZ	Metronidazole
NI	Nitroimidazole
IgA	Immunoglobulin A
NHPs	Nonhuman primates
NIAID	National Institute of Allergy and Infectious Diseases
CT	Computed tomography
ALA	Amoebic liver abscess
CNS	Central Nervous System
µm	Micrometer
LAMP	Loop-mediated isothermal amplification assay
HLY6	Hemolysin 6

SREHP	Serine-rich *Entamoeba histolytica* protein
Gal/GalNAc	Galactose/N-acetyl galactosamine
EIA	Enzyme Immunoassay
STRs	Short tandem repeats
PVM	Parasitophorous vacuole membrane
AIDS	Acquired immune deficiency syndrome
IVDUs	Intravenous drug users
GBD	Global Burden of Disease
GEMS	Global Enteric Multicenter Study
NTD	Neglected tropical Disease
PRV	priority review voucher
UV	Ultraviolet
H_2SO_4	Sulfuric acid
DIC	Differential interference contrast
ICT	Immunochromatographic dipstick test
mAbs	Monoclonal antibodies
RFLP	Restriction fragment length polymorphism
MAS-PCR	Multiplex allele-specific PCR
TRAP C1	Tumor necrosis factor receptor-associated protein 1
COWP	*Cryptosporidium* oocyst wall protein
Hsp70	Heat shock protein 70
DHFR	Dihydrofolate reductase
GP60	Glycoprotein 60
HAART	Highly active antiretroviral therapy
NTZ	Nitazoxanide
CDC	Center for Disease Control and Prevention
LSU rRNA	Large subunit Ribosomal ribonucleic acid
KGy	Kilogray
HPP	High Pressure Processing
MPa	Megapascal
NaDCC	Sodium dichloroisocyanurate
TMP–SMX	Trimethoprim–sulfamethoxazole
Mg	Milligram
KI	Potassium iodide
ml	Milliliter
µl	Microliter
µM	Micromole
$MgCl_2$	Magnesium chloride
mM	Milliliter
U	Unit
rTaq	Recombinant Taq
ng	Nanogram
dNTPs	Deoxynucleoside triphosphate
pmol	Picomole
bp	Basepair
PC	Positive control

NC	Negative control
M	Molar
Rpm	Revolution per minute
BLAST	Basic Local Alignment Search Tool
ML	Maximum Likelihood
MEGA	Molecular Evolutionary Genetics Analysis
OR	Odds Ratio
CI	Confidence Interval
SNP	Single Nucleotide Polymorphism
RRmix	Ready reaction mix
min	Minute
sec	Second
DnaSP	DNA Sequence Polymorphism
PopART	Population Analysis with Reticulate Trees
S	variable site
Hd	Haplotype diversity
H	Haplotype
π	nucleotide diversity
K	Average nucleotide differences
SD	Standard Deviation
ST	Stool type
ND	Non diarrheal
SD	Sole diarrheal
DC	Diarrheal coinfection
LD	Linkage Disequilibrium
R_m	Number of recombination event
MLST	Multilocus sequence typing
dsDNA	Double stranded deoxyribonucleic acid
pg	Picogram
µg	Microgram
Se	Sensitivity
Sp	Specificity
Ac	Accuracy
PPV	Positive predictive value
NPV	Negative predictive value
κ	Kappa statistics
LDL	Lower Detection Limit
TP	True positive
FP	False positive
TN	True negative
FN	False negative
PPA	Positive percentage agreement
NPA	Negative percentage agreement
OA	Overall agreement
HCMP	High Cysteine Membrane Proteins
NR1	Nitroreductase 1

NR2	Nitroreductase 2
ENO	Enolase
ng/µL	Nanogram per microliter
µg/ml	Microgram per milliliter
sSNPs	Synonymous single nucleotide polymorphism
nsSNPs	Non synonymous single nucleotide polymorphism
SNVs	Single nucleotide variants
nsSNVs	Non synonymous single nucleotide variants
CDS	Coding sequence

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Thesis Title: Detection of common enteric parasites in Kolkata and characterization of the pathogenic factors of local isolates of *Giardia lamblia*

Abstract:

Background: Enteric parasitic infections remain a significant public health concern, particularly in regions with favorable environmental conditions for pathogen transmission. This study aimed to investigate the prevalence, genetic diversity, and clinical implications of enteric protozoan parasites, with a focus on *Giardia lamblia*, among diarrheal patients and asymptomatic individuals in eastern India. A cross-sectional study was conducted in the eastern region of India. Human fecal samples were collected from diarrheal patients and asymptomatic individuals from different community. Microscopic and molecular assays were performed to identify common enteric protozoan parasites. *Giardia lamblia* isolates were subjected to multilocus sequence typing (MLST) analysis using recommended genetic markers. A novel multiplex PCR-RFLP approach was developed for simultaneous detection and differentiation of G. lamblia assemblages and sub-assemblages. Additionally, genetic variability of virulence genes and genes involved in Metronidazole (MTZ) metabolic pathways was investigated. The overall prevalence of intestinal parasitic infections among diarrheal patients was 18.56%. *Giardia lamblia* (6.67%) emerged as the most prevalent protozoan, followed by *Entamoeba* spp. (4.52%), *Cryptosporidium* spp. (4.17%), and *Cyclospora cayetanensis* (3.18%). Parasitic infections showed seasonal trend, with higher rates during humid rainy seasons compared to dry winter months. Genotyping of *G. lamblia* isolates revealed two distinct assemblages: *Giardia enterica* (assemblage B) and *Giardia duodenalis* (assemblage A). Assemblage B was twice as prevalent as assemblage A. Within assemblage A, subtype AII was more common than AI. A potential correlation between assemblage A and symptomatic giardiasis was observed. The community-based survey of asymptomatic individuals revealed a higher prevalence of *G. lamblia* (10.3%) compared to symptomatic cases. Asymptomatic carriage was notably prevalent in the 2-20 years age group. Assemblage B remained the dominant genotype in asymptomatic cases, consistent with findings from symptomatic co-infections. The developed multiplex PCR-RFLP method demonstrated high diagnostic sensitivity (94.2%), specificity (100%), and accuracy (97.1%) for giardiasis diagnosis. This approach enabled simultaneous identification and differentiation of assemblages A and B, as well as subtyping of AI, AII, BIII, and BIV. Genetic analysis of virulence genes and MTZ metabolic pathway genes revealed higher allelic diversity and non-synonymous single nucleotide variations (SNVs) in assemblage B compared to assemblage A. This genetic diversity may contribute to the adaptability and MTZ inactivation capability of assemblage B parasites. This study provides comprehensive insights into the epidemiology and genetic characteristics of enteric protozoan parasites, particularly *G. lamblia*, in eastern India. The high prevalence of *G. lamblia* in both symptomatic and asymptomatic populations underscore its public health importance. The dominance of assemblage B and its extensive genetic variability suggest potential implications for parasite adaptability and drug resistance. The observed genetic diversity in virulence and MTZ metabolism genes highlights the need for continued surveillance of drug resistance and the development of alternative treatment strategies.

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Chapter 1

INTRODUCTION

Infections affecting the gastrointestinal tract, caused by a range of bacterial, parasitic, and viral pathogens, can profoundly disturb the normal functioning of the intestines, often resulting in diarrhea. Diarrhea can often not be clinically differentiated based on the specific causative agent. However, the introduction of glucose-electrolyte oral rehydration therapy (ORT) has had a remarkable impact on reducing acute mortality resulting from dehydration due to diarrhea. Global mortality related to diarrhea have decreased significantly, declining from around 4.6 million annual deaths in the 1980s to the current estimated range of 1.6–2.1 million deaths (Keusch, et al. 2006). Despite the decline in mortality rates from diarrhea, morbidity rates associated with this condition remain persistently high. Morbidity stemming from malnutrition, which can result from prolonged diarrhea and enteropathy caused by chronic and recurring enteric infections, is often not considered in estimation of the overall burden of diarrhea (Petri et al., 2008). The prevalence of diarrheal diseases is closely tied to a country's economic condition, particularly by affecting the health and productivity of its workforce. Recent studies indicate that the potential disability-adjusted life year (DALY) impact of morbidity resulting from diarrhea is even more significant than the substantial mortality associated with this condition. Diarrheal diseases consistently rank among the top four factors contributing to years of life lost in regions like South Asia and sub-Saharan Africa. The majority of these deaths are observed in children under the age of 5 years, primarily from developing nations (WHO, 2024). The absorptive function of a healthy intestinal tract plays a crucial role during the early formative years of life. Significant development of the human brain and synapses occurs within the first two years after birth and it is of utmost importance during this period to absorb essential nutrients through our gut to ensure the optimal growth and development of the body (Petri et al., 2008). The impact on morbidity from enteric pathogens is associated with their capacity to directly impede intestinal absorption and their ability to induce diarrhea, both of

which have adverse effects on nutritional status of a child. Diarrhea is a significant contributor to child mortality, responsible for about 15% of all child deaths under the age of five. In certain regions, such as South East Asia and Africa, this percentage is even higher, with rates reaching approximately 31% and 25%, respectively. It also remains a significant cause of adult mortality as well in these regions (Walker et al., 2012).

Diarrhea can manifest in three distinct clinical syndromes: acute watery diarrhea, dysentery, and persistent diarrhea. Acute watery diarrhea typically occurs for a duration of several hours to a few days, whereas persistent diarrhea is a type that endures for 14 days or longer. In contrary, dysentery is a condition characterized by acute bloody diarrhea with mucoid stool (WHO, 2024). Each syndrome has a different underlying cause and requires specific treatment approaches. Apart from bacteria and viruses, parasites, especially in developing countries, play a significant role in causing diarrhea.

Enteric parasites continue to play a significant role in the occurrence of diarrhea, affecting populations in both developing and developed nations (Hodges & Gill, 2010). It is important to note that parasitic infections can sometimes be overlooked, leading to an incomplete understanding of their causes over time. Diarrhea-causing parasitic protozoa found in the intestinal tract include *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* spp. and *Cyclospora cayetanensis*. These organisms are responsible for amoebiasis, giardiasis, and cryptosporidiosis and are commonly associated with diarrheal illnesses, especially in developing regions like Africa, South Asia etc. In contrast, enteric parasites like *Blastocystis* spp. and *Dientamoeba fragilis* seem to be more prevalent in developed countries (Nair et al. 2010; Fletcher et al. 2011; Roberts et al. 2011; Fletcher et al. 2012).

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, leading to a diverse population with varying socioeconomic conditions. These factors create a favorable environment for the proliferation of enteric pathogens, increasing the likelihood of encountering numerous opportunistic enteric parasites in this region (Mukherjee et al., 2009). However, there is limited information available regarding the diversity of the species infecting individuals in this area. Kolkata continues to face a significant number of patients afflicted by diarrhea, which poses concerns not only for the city but also for eastern India as a whole, leading to indirect economic implications. Recent hospital-based data from the region on diarrheal morbidity has revealed a significant presence of parasitic infections. Notably, asymptomatic infections are also prevalent in this population. Studies conducted in this region have shown a substantial prevalence of *Giardia lamblia, Entamoeba histolytica*, and *Cryptosporidium* spp. (Mukherjee et al., 2009; Das et al. 2014). However, these studies alone are insufficient to accurately estimate the burden of parasitic infections and understand the current trends of diarrheal parasitic infections. Therefore, it is highly recommended to properly identify and characterize the parasites to enhance prevention and control measures for parasitic diarrheal cases. Comprehensive efforts in identifying and understanding the specific parasites involved are crucial for the effective management and mitigation of parasitic-related diarrhea.

The Beliaghata Infectious Disease Hospital and B. C. Roy Children Hospital consistently witness a steady influx of diarrhea 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 diarrhea. This has enabled the undertaking of the present study, which aims to enhance our understanding of the issue.

The objective of this study is to assess the prevalence of common enteric parasites among patients suffering from diarrhea in Kolkata and its surrounding areas. Descriptive scientific studies addressing the prevalence and seasonal distribution of *Entamoeba* species, *Cryptosporidium* species, *Cyclospora cayetanensis* and *Giardia lamblia* in and around Kolkata have not been conducted, thus limiting our comprehensive understanding of their occurrence. Given the public health importance of intestinal protozoa infections, the present study seeks 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 as well as through community-based surveillance. 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.

The study objectives are organized below-

1. **Detection of the common enteric protozoan parasites from diarrheal stool samples in Kolkata and adjacent areas by conventional microscopy and molecular methods.**
2. **To create a preliminary database on the incidence of this enteric protozoan infection in the study area.**
3. **Genotyping and sub-genotyping of local isolates of *Giardia lamblia* found in study population**
4. **Molecular Analysis of virulence factors of the isolates found.**

The following chapters fulfill the stated objectives-

- **Chapter 3: Detection of the enteric protozoan parasites from diarrheal stool samples obtained in Hospital based surveillance (Objective 1 and 2)**
- **Chapter 4: Genotyping of diarrhea causing *Giardia lamblia* local isolates (Objective 3)**
- **Chapter 5: Genotyping of *Giardia lamblia* local isolates from asymptomatic population through Community based surveillance (Objective 3)**
- **Chapter 6: Development of a rapid and cost-effective Multiplex PCR-RFLP method for genotyping of *Giardia lamblia* (Objective 3)**
- **Chapter 7: Genetic characterization of the potential virulence factors of the *Giardia lamblia* local isolates (Objective 4)**

Chapter 2

Review of Literature

Giardia Species

Introduction

Giardia is a unicellular flagellated eukaryotic microorganism. This microbe is particularly notorious for triggering waterborne-foodborne diarrheal outbreaks of parasitic origin in wide range of vertebrate hosts (Adam 2001). The genus *Giardia* exhibits a wide host range, infecting a variety of hosts, from mammals to amphibians and birds. Previous taxonomic investigations have recognized only six discrete *Giardia* species, among which *G. agilis, G. ardeae, G. muris, G. microti,* and *G. psittaci* primarily parasitize various animal species. *G. muris* predominantly infects rodents, and *G. agilis* is typically associated with amphibians. In contrast, *G. lamblia* (syn *G. duodenalis, G. intestinalis*) has the unique ability to infect both humans and a broad spectrum of mammalian hosts, including pets, livestock and wildlife (Feng and Xiao, 2011). Recently, there has been emerging evidence pointing to the potential existence of a separate *Giardia* species in reptiles. This evidence stems from the identification of a parasite resembling *G. lamblia* in lizards. However, this particular parasite displayed significant distinctions, such as the absence of median bodies and the presence of binucleated cysts. Consequently, it has been classified as a unique *Giardia* species and named *G. varani* (Upton and Zien, 1997.). Wild freshwater and marine fish in Australia have indicated the presence of zoonotic genotypes of both *G. lamblia* and *G. microti.* It remains unclear whether these fish serve as primary hosts for these *Giardia* species or act as vectors for the transmission of *Giardia*. Notably, *G. psittaci* is identified in parakeets, and *G. ardeae* is found in herons. Furthermore, *G. microti* has been proposed as a distinct species due to its host specificity for voles and muskrats (Yang et al., 2009). Consequently, the host range of *Giardia* species varies significantly, with *G. lamblia* having the most extensive host range, making

it of paramount public health significance. Consequently, giardiasis is classified as a zoonotic disease.

The species of *Giardia* can be visually differentiated from one another through the use of light microscopy. In cases where two species closely resemble each other, subtle differences may be discerned with the aid of electron microscopy. In 1952, Filice presented an extensive morphological analysis of *Giardia* and recommended the usage of three species names based on the characteristics of the median body: *G. lamblia, G. muris*, and *G. agilis* (Filice, 1952). *G. lamblia* exhibits a pear-shaped morphology and typically possesses one or two transverse, claw-shaped median bodies. In contrast, *G. agilis* is elongated and slender, with a teardrop-shaped median body, as observed in light microscopy. The trophozoite of *G. muris* is characterized by its distinct features, such as being shorter and rounder, with a small, rounded median body. For *Giardia* isolates initially grouped with *G. lamblia* based on morphological characteristics distinguishable through light microscopy, electron microscopy has revealed differences significant enough to warrant the description of additional species. *G. microti* exhibits distinctions in cyst morphology as assessed by electron micrography, and differences in the 18S rRNA sequences when compared to *G. lamblia* of human origin (Adam 2001). Given that *G. lamblia* is the primary causative agent of giardiasis in humans, as well as a pathogen in pets, livestock, and wildlife, comprehensive scientific research on this organism is imperative.

Taxonomy

The Protozoa is classified into seven phyla in the commonly used 1980 classification. *Giardia* is a member of the Phylum Sarcomastigophora, Subphylum Mastigophora (Flagellata), Class Zoomastigophorea, Order Diplomonadida, and Family Hexamitidae in the old systematic based

on morphology (Morrison et al., 2007). In the new systematic based on genetic, structural, and biochemical data *Giardia* is classified as belonging to the Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplobozoa, Order Giardiida, and Family Giardiidae (Cavalier-Smith, 2003).

Kingdom: Protista

Sub-Kingdom: Protozoa

Phylum: Metamonada

Subphylum: Trichozoa

Superclass: Eopharyngia

Class: Trepomonadea

Sub-Class: Diplobozoa

Order: Giardiida

Family: Giardiidae

Genus: *Giardia*

Giardia is a highly peculiar, ancient-looking eukaryotic single-celled organism that resembles anaerobic prokaryotes in many ways. Common eukaryotic subcellular components like mitochondria, peroxisomes, and presumably a conventional golgi apparatus are absent from *Giardia*. Developmentally controlled formation of large secretory compartments containing cyst-wall material takes place during encystation. All known anaerobic, amitochondrial protist groups are thought to have evolved through the loss of their mitochondrial genomes, cytochromes, and oxidative phosphorylation, as well as the transformation of their mitochondria into double-

membraned organelles like mitosomes, according to molecular evidence (Cavalier-Smith, 2003; Dolezal et al., 2005). *Giardia* emerged somewhat later than *Trypanosoma* and *Euglena* during the early evolution of Excavata eukaryotes, according to a comparison of small nucleolar RNAs identified from Archaea and unicellular eukaryotes (Luo et al., 2009).

Giardia lamblia

Giardia lamblia, also known as *Giardia duodenalis* and *Giardia intestinalis*, is a species within this genus responsible for giardiasis in both humans and the majority of mammalian species. *Giardia* was first observed by Van Leeuwenhoek in 1681 while examining his own diarrheal stools through a microscope. Later in 1859, Lambl a more detailed description of the organism was provided. Lambl initially classified the organism under the genus *Cercomonas* and named it *Cercomonas intestinalis*. Subsequently, some researchers named the genus after Lambl, while others named the human form of the species after him, leading to the nomenclature *G. lamblia*. The species names *Giardia lamblia, Giardia duodenalis* and *Giardia intestinalis* are commonly used interchangeably nowadays, all referring to the same microorganism.

Life Cycle

Giardia has two stages in its life cycle: 1) The resistant cyst stage; 2) the vegetative trophozoite stage. The environmentally stable cyst stage makes it easier for transmission of the parasite when feces from one host is ingested by a subsequent host. The cyst has a two-layered cyst wall and measures 5 μm × 7–10 μm. The primary sugar present in the cyst wall is 7–20 nm-long filaments that cover the outer filamentous layer is N-acetylgalactosamine. The cyst stage is distinguished by the presence of four nuclei, in contrast to the two nuclei observed in trophozoites (Fig. 2.1). With a metabolic rate ranging from 10% to 20% of that exhibited by the trophozoite, the cyst enables

prolonged survival in the environment, particularly in cool and moist settings. This disparity in environmental resilience may contribute to the elevated incidence of giardiasis in endemic areas (Adam, 2021).

The trophozoites of *G. lamblia* have a pear-shaped body, measuring 8µm in width and 12 to 15µm in length. Their concave ventral side, encircled by the lateral crest and a flange (Fig. 2.1), enables the organism to adhere to the intestinal epithelium. They also possess four pairs of flagella. Two symmetrically positioned nuclei are located in the anterior half of the organism, and the dorsal surface is convex.

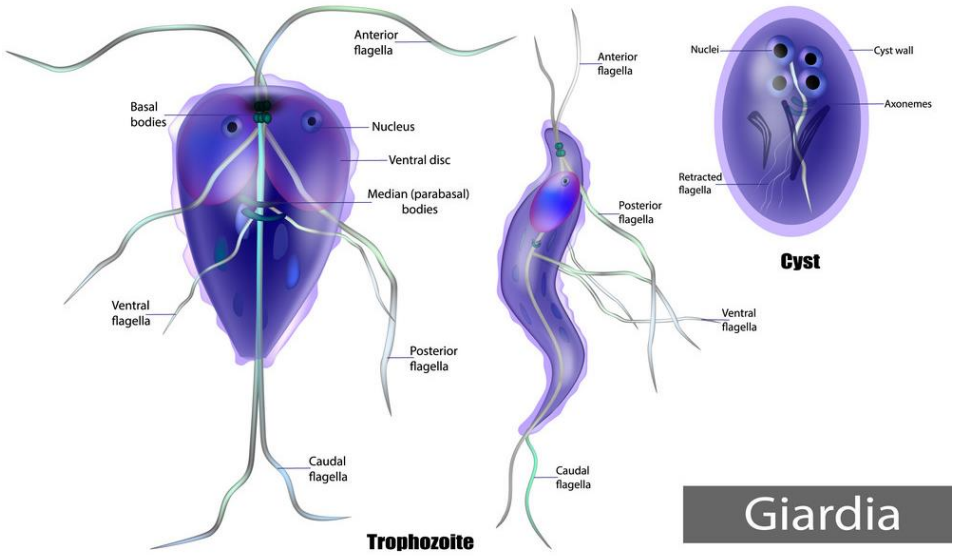


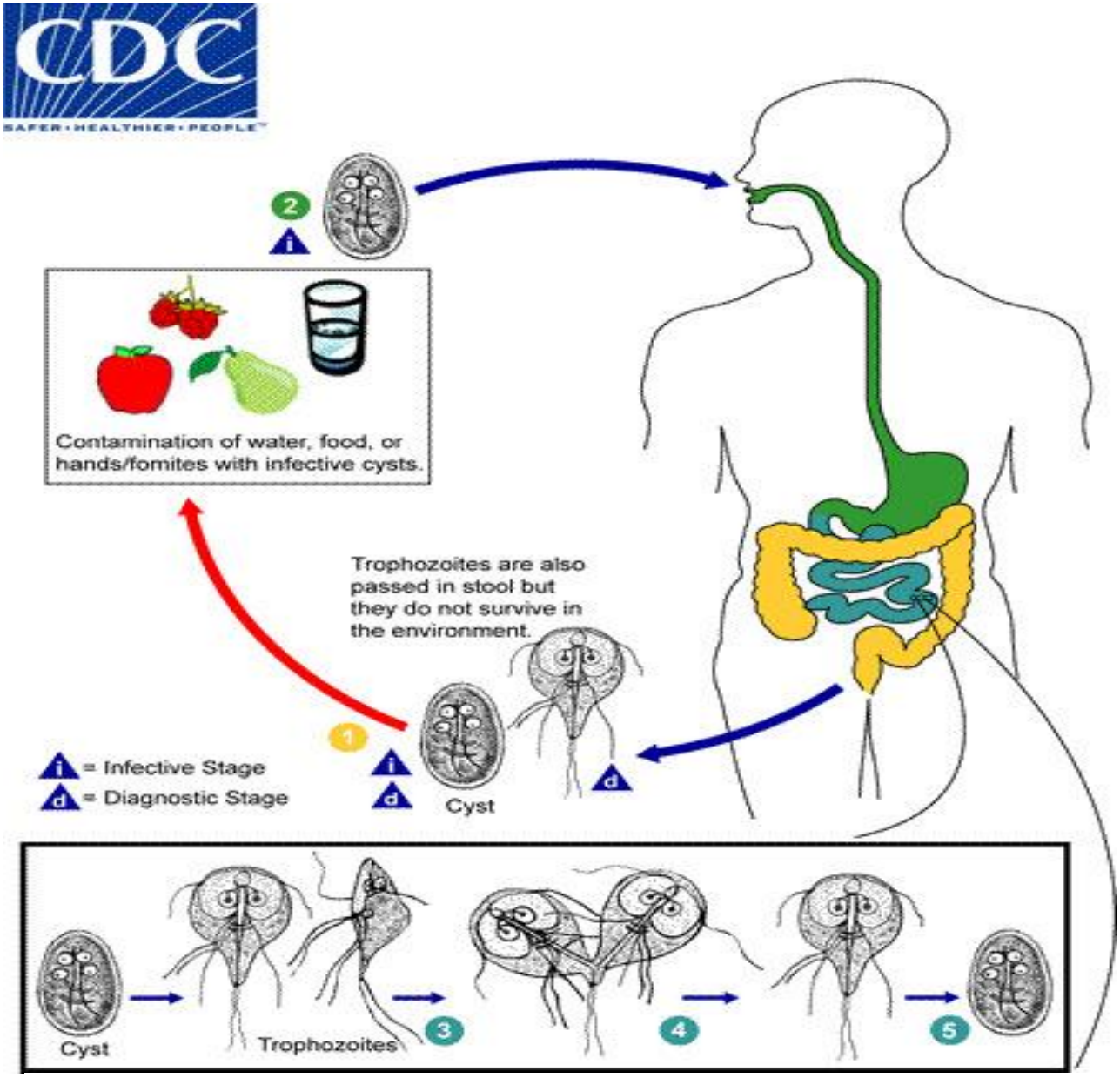
Fig. 2.1: Morphology of Trophozoite and Cyst stage in life cycle of *Giardia lamblia*

Infection begins after food or water contaminated with cysts are consumed and they travel from acidic environment of the stomach into the duodenum (Fig. 2.2). The cysts are excysted when exposed to bile and an alkaline pH. *Giardia* can, however, also be excysted at a neutral pH (Feely et al., 1991), and people who have had their stomach pH raised artificially or by nature are vulnerable to *Giardia* infection (Cook, 1985). As indicated by a report of two cases of gastric

giardiasis in patients treated with a proton pump inhibitor and hypochlorhydria, they may actually be more vulnerable to chronic giardiasis (Reynaert et al., 1995). The trophozoites multiply in the small intestine, where some of them develop into cysts.

Encystation begins shortly after infection and reaches a peak within a week. In the clusters of increased organism density, encystation starts to occur (Barash et al., 2017). The induction step of encystation requires presence of bile salts, bile, and an alkaline pH of 7.8 (Boucher & Gillin, 1990). Trophozoites undergo a transformation, adopting a rounded shape, and certain essential cytoskeletal components of the trophozoite are disassembled during encystation (Palm et al., 2005).

In this phase, chromosome replication occurs twice and one nuclear division, culminating in the formation of a mature cyst with four nuclei, each of which is 4n. Subsequently, under suitable conditions in host gut environment, an aperture at one pole of the cyst permits the flagella and excyzoite cell body to emerge. The excyzoite cell body then divides twice, producing four trophozoites, each of which has two diploid nuclei (Bernander et al., 2001).



Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in the feces (diagnostic stages) ❶. 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) ❷. In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites) ❸. 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 ❹. Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in nondiarrheal feces ❺. 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.

Fig 2.2: Life cycle of *Giardia lamblia*

Giardiasis: Clinical Manifestation

As a non-invasive parasite *Giardia* infects the small intestine, establishing itself in the lumen and on the epithelial surface (Certad et al., 2017). Trophozoites attach to the epithelial cells, leading to microvilli shortening. This attachment targets specific signaling networks, activating apoptosis, causing the loss of intercellular junctions, cytoskeletal rearrangement, and barrier dysfunction, ultimately contributing to diarrhea (Allain and Buret, 2020). While *G. lamblia* trophozoites are typically found in the proximal small intestine, they have also been identified in the stomach, distal small intestine, caecum, and pancreas (Halliez and Buret, 2013). Although asymptomatic infections are common, a variety of symptoms, including diarrhea, bloating, epigastric pain, nausea, and vomiting, may occur after a 1–2 weeks incubation period (Allain and Buret, 2020; Certad et al., 2017). Young children are particularly susceptible to developing symptoms (Thompson and Ash, 2016). Despite being considered a significant cause of diarrhea, the role of diarrhea in giardiasis has been debated due to the high frequency of asymptomatic infections (Leung et al., 2019; Ryan et al., 2019). Some evidence suggests that *Giardia* might even provide protection against diarrhea caused by concurrent infections with other enteric pathogens (Allain and Buret, 2020). The acute phase of giardiasis typically lasts 1–3 weeks, but symptoms may persist for months. Although most infections resolve on their own, recurrence is common. Chronic infections can lead to malnutrition, micronutrient deficiencies, malabsorption, weight loss, and are linked to impaired growth and cognitive development in young children (Allain and Buret, 2020; Certad et al., 2017). Post-infectious complications encompass irritable bowel syndrome, functional dyspepsia, and chronic fatigue syndrome, while extra-intestinal complications may include lower cognitive function, ocular pathologies, arthritis, allergies, and others (Allain and Buret, 2020).

The clinical manifestations of human giardiasis encompass a wide spectrum, ranging from brief, mild, and transient intestinal issues that completely resolve, to a more distinctive set of symptoms. This includes an abrupt onset of diarrhea, abdominal cramps, bloating, and flatulence, often accompanied by nausea and weight loss lasting for up to seven weeks. Some instances of giardiasis are characterized by episodic symptomatic periods recurring over several years. An inherent feature of *Giardia* infections in any host is the intermittent shedding of cysts. In undernourished individuals and children, the infection can become chronic, leading to profound diarrhea, weight loss, disruption of absorption, and growth issues (Lebwohl et al., 2003). Moreover, individuals may be infected without experiencing relevant symptoms, and there's even a suggestion that some individuals may benefit from their carrier state. For instance, healthy day care children with asymptomatic *G. lamblia* infection displayed no disadvantage and possibly even advantages in nutritional status and freedom from other illnesses (Ish-Horowicz et al., 1989). The reasons why some individuals develop clinical giardiasis while others remain asymptomatic are not fully understood. However, factors such as immune status, nutritional status, age, concurrent enteric infections, environmental factors, and variations in the virulence and pathogenicity of *G. lamblia* strains likely contribute to the severity of the infection (Thompson, 2004).

Transmission

Giardiasis is spread through the faecal-oral route and can be waterborne or foodborne, or it can be direct (animal to animal, person to person, or zoonotic). Person-to-person transmission has been extensively documented as a major source of infection, especially in institutional settings such as day care centers and nursing homes where personal hygiene may be subpar (Adam et al., 2016). There has also been evidence of giardiasis transmission through sexual contact (Escobedo et al., 2014). There has been a lot of focus on the potential for zoonotic transmission, especially from

cattle, dogs, and cats, since many different mammals can also harbor *G. lamblia*, frequently including Assemblages A and B (Thompson and Ash, 2016). However, there is little direct evidence to support zoonotic transmission of *G. lamblia*. (Xiao and Feng, 2017; Ryan and Zahedi, 2019). It is also worth noting that the presence of common genotypes in humans and companion animals does not prove zoonotic transmission (Feng and Xiao, 2011). The high frequency of *G. lamblia* infections in livestock and the existence of zoonotic assemblages, despite the lack of direct evidence, raise concerns about the possibility of transmission to humans through direct contact (such as farmers, veterinarians, and animal handlers) or indirect contact (such as food and water contamination) (Santin, 2020). It has also been suggested that humans can contract reverse zoonotic transmission or zooanthroponosis, particularly from companion animals or wildlife (Heyworth, 2016; Thompson and Ash, 2016). Since many outbreaks have been linked to drinking water contaminated with cysts, the waterborne route is likely the most well-known means of giardiasis transmission (Ryan et al., 2019). There have also been an increasing number of reports of illness outbreaks linked to recreational water (Adam et al., 2016; Xiao and Feng, 2017). Foodborne transmission of giardiasis is now becoming more widely known, despite being far less common than other routes. According to importance, *G. lamblia* ranked eleventh out of all food-borne parasites worldwide (FAO/WHO, 2014). Foodborne illness has been linked to changes in consumer behavior, international travel, and the growing globalization of the food trade (Dixon, 2016).

Genome

Trophozoites have two diploid nuclei and are tetraploid, meaning they have a ploidy of four. The size of the five chromosomes ranges from roughly 1 to 5 Mb (Adam et al., 1988). With few noncoding regions, the entire genome is compact, weighing in at around 12 megabytes. Eight cis-

spliced and five trans-spliced introns have been identified, indicating the rarity of introns (Xue et al., 2018; Xu et al., 2020). 2,099 of the 4,863 protein-coding genes in the genome are hypothetical proteins; the remaining genes are annotated. Furthermore, 306 pseudogenes are present. Noncoding small RNAs (sRNAs) have also been found throughout the genome. These include tRNA-derived sRNA (Liao et al., 2014), endogenous siRNA (endo-siRNA), and microRNAs (miRNA) (Saraiya et al., 2014). They are thought to reproduce in which case, there would be an expectation of increasing allelic heterozygosity over time. However, their genome shows an extremely low level of allelic heterozygosity (Adam, 2021).

Molecular characterization

In the epidemiological investigation of *G. lamblia*, molecular genotyping is essential for tracking the sources of contamination (Cacciò and Ryan 2008). *G. lamblia* is thought to represent a species complex because of its exceptional genetic diversity and low morphological variation. According to reports, eight different genetic assemblages (A–H) have been identified, with assemblages A and B being important human pathogens. These two assemblages are present in a wide range of mammals and have a wider host range (Takumi et al. 2012). It has been observed that the assemblages C–H have smaller host ranges and sporadically infect humans (Table 2.1.) (Cacciò and Sprong 2010; Mirdha et al. 2014; Fantinatti et al. 2020).

Table 2.1. *Giardia lamblia* assemblages and host range

Assemblages	Hosts	Proposed species name
AI	Primarily animals, but also in humans	*Giardia duodenalis*
AII	Humans, numerous other mammals	*Giardia duodenalis*
B	Humans, numerous other mammals	*Giardia enterica*
C	Dogs	*Giardia canis*
D	Dogs	*Giardia canis*
E	Cows, sheep, alpacas, goats, pigs	*Giardia bovis*
F	Cats	*Giardia cati*
G	Rats and mice	*Giardia simondi*
H	Seals (marine vertebrates)	NA

Highly discriminative multilocus sequence typing is used to examine the genetic variation levels within assemblages. Partial sequences of three conserved housekeeping genes—glutamate dehydrogenase (*gdh*), β-giardin (*bg*), triose phosphate isomerase (*tpi*), and small subunit ribosomal ribonucleic acid (SSU rRNA)—are frequently examined for *G. lamblia* subtyping. Following phylogenetic analysis, the closely related genotypes were grouped into assemblages and subassemblages. Research conducted globally has demonstrated that assemblage A and B distributions vary geographically (Stark et al. 2009).

The primary lineages of human-derived *Giardia* were previously assigned to a different species (*G. lamblia*): All of the human isolates are contained in Mayrhofer's (1995) de-identified assemblages A and B, which align with groups I plus II and III plus IV (Andrews et al., 1998; Karanis and Ey, 1998), Polish and Belgian genotypes (Homan, 1992), and groups 1 plus 2 and 3

(Nash and Keister, 1985). Although some isolates of *G. lamblia* derived from animals resemble or are identical to specific genotypes derived from humans, others represent distinct genotypes that are likely host specific. WB strain clone C6 (AI), DHA2 (AII), and GS (B) genome sequence data can be found online in the *Giardia* genome project database (McArthur et al., 2000). It was discovered that in protein-coding regions, the genomes of the WB and GS strains exhibit 77% nucleotide and 78% amino acid identity (Franzén et al. 2009). The two isolates have entirely different variable surface protein (VSP) repertoires, and comparative analysis revealed 28 unique GS and 3 unique WB protein coding genes. The binding sites for transcription factors specific to encystation are absent from the promoters of multiple enzymes involved in the synthesis of the cyst-wall in GS. Numerous syntony breaks were found and confirmed (Plutzer et al., 2010). All of the genes were found to be capable of reproducibly grouping isolates into their assemblages, and A-I and A-II subassemblages were robust and identifiable at all loci (Wielinga and Thompson 2007). Assigning assemblage B has occasionally been difficult using four markers. The existence of A-I, A-II, and a new A-III subassemblages was supported by multilocus sequence and phylogenetic analysis. Levels of genetic diversity in assemblage B were greater than those found in assemblage A, with many of the subassemblages representing single isolates from specific hosts. In assemblage B isolates, however, variable levels of intra-isolate sequence heterogeneity have been reported. However, no evidence of intra-isolate sequence heterogeneity was found for assemblage A isolates (Caccio et al., 2008). According to reports, multilocus genotyping is a highly discriminatory tool for determining zoonotic sub-groups within assemblage A, but it is less useful for subtyping assemblage B due to the high frequency of double peaks in the sequence chromatograms (Lebbad et al., 2009). The majority of the assemblage B isolates were unable to be classified into previously described subassemblages. When polymorphisms from the three loci

were combined, isolates could only be assigned to a specific group (Levecke et al., 2009). Mixtures of genotypes were repeatedly observed in individual isolates, making multilocus genotyping (MLG) more difficult and complex (Sprong et al., 2009).

Epidemiology

The prevalence of giardiasis ranges from 2% in developed nations to as high as 70% in developing countries (Júlio et al., 2012; Fletcher et al., 2013). The elevated rates of the disease in developing countries can be attributed to various local risk factors such as insufficient basic sanitation and hygiene facilities, limited access to safe drinking water, and substandard housing conditions (Fletcher et al., 2013). However, it is probable that the true number of endemic giardiasis cases in developed countries is underestimated, particularly because individuals returning from travel are more likely to undergo testing for *G. lamblia* infection compared to those without a history of travel (Zajaczkowski et al., 2018). According to a study conducted in the USA, 17.7% of HIV-positive patients had giardiasis (Hooshyar et al. 2019). Recent studies have indicated infection rates of 4.0% in Belgium, 1.5% in Germany, 0.4% to 6.2% in Italy, 3.7% in Portugal, 5.4% in Spain, 1.3% in the United Kingdom, 1.4% in the United States, 1.1% to 6.6% in Saudi Arabia, 2.5% in South Korea, 1.6% to 7.6% in Australia, and 7.6% in New Zealand (Feng Y and Xiao L. 2011). In a study conducted in Ujjain, Madhya Pradesh, India, the prevalence of *G. lamblia* in 5990 samples over a five-year period was reported to be 3.9% (Marothi & Singh, 2011). Another investigation in Kolkata in 2008, conducted through a year-long hospital-based survey revealed a prevalence of approximately 13% (Mukherjee et al., 2009). Many of the people whose stool samples contain *Giardia* are asymptomatic; in fact, several studies have found that asymptomatic people's stool often contains *Giardia* more often than that of people who have acute diarrhoea. Even in the absence of acute diarrhoea, giardiasis may be linked to stunted growth in children in

environments with poor resources (Leder and Weller, 2020). In the UK, a study revealed a notable occurrence of asymptomatic *Giardia* infection. The study identified an additional *Giardia* infection in 30% of households, and within these households, 17% of all contacts were found to have asymptomatic infection (Waldram et al., 2017). In a separate investigation in the Zaria area of Kaduna, Nigeria, researchers reported a prevalence of 41.4% for asymptomatic giardiasis in children (Helen et al., 2011). Despite the significant prevalence of *Giardia*, there is limited information on variations in virulence and clinical manifestations among different genotypes. Due to its high prevalence in tropical regions and recurrent infections with an average duration of persistent cyst shedding for more than 6 months in some cases, giardiasis was added to the Neglected Tropical Disease Initiative (NTDI) of World Health Organization (WHO) in 2004 (Júlio et al. 2012; Bartelt et al. 2013). Giardiasis, affecting approximately 200 million people globally each year, leads to around 500,000 deaths, with children aged <5 years being the most heavily impacted (Feng Y and Xiao L. 2011). Since the beginning of the previous century, more than 100 outbreaks of waterborne giardiasis have been documented globally (Karanis et al., 2007). In past years, there have been reported outbreaks of giardiasis associated with interactive water fountains in Florida (USA) and with drinking water in New Hampshire (USA) and Nokia (Finland) (Daly et al., 2010; Rimhanen-Finne et al., 2010; Eisenstein et al., 2008). The most significant drinking water-related outbreak to date occurred in Norway in 2004, affecting approximately 1500 individuals (Robertson et al., 2006).

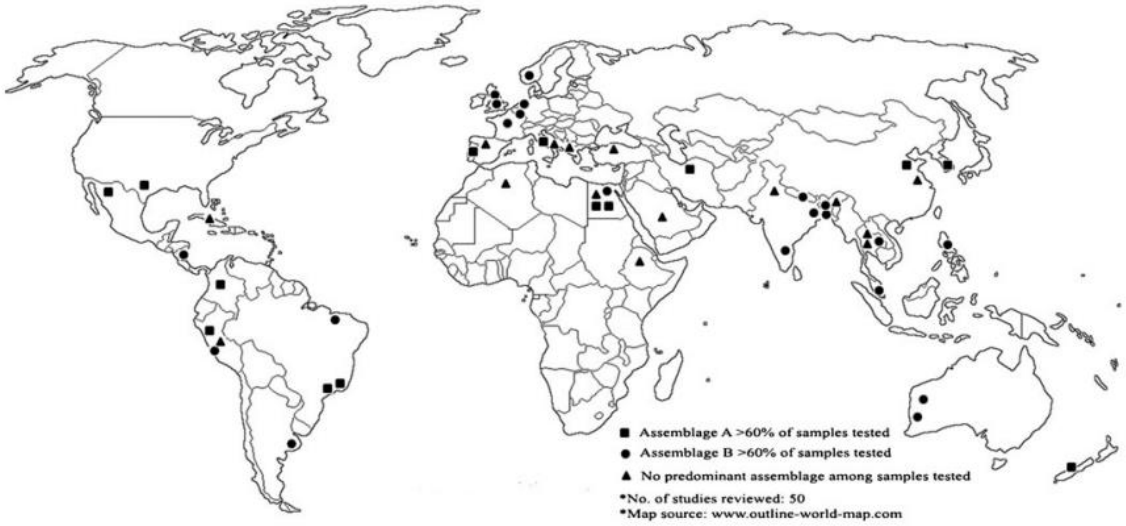


Fig 2.3: Global distribution of *Giardia lamblia* assemblages A and B

There is considerable uncertainty regarding the global distribution of *G. lamblia* assemblages due to the limited number of molecular epidemiological studies on giardiasis in humans. Although both *G. lamblia* assemblages A and B are found worldwide, studies conducted in developed and developing regions suggests that assemblage B is the predominant one in human infections (Breathnach et al., 2010). Notably, Canada (Iqbal et al., 2015), China (Yu et al., 2019), England (Minetti et al., 2015a), Kenya (Mbae et al., 2016), and Spain (Wang et al., 2019) report higher prevalence of assemblage B. Assemblage B infections exhibit greater parasitic load and cyst shedding, likely contributing to their higher detection rates compared to assemblage A infections (Kohli et al., 2008). However, certain countries like Brazil (Souza et al., 2007), Ethiopia (Damitie et al., 2018), Iran (Mahmoudi et al., 2020), New Zealand (Winkworth et al., 2008), Syria (Skhal et al., 2017), and Romania (Costache et al., 2020) report higher rates of assemblage A infections.

Recent research has suggested that mixed-assemblage infections are also more prevalent in developing countries, possibly due to crowded living conditions and increased contact among

infected individuals, providing an environment for both assemblages A and B to persist and circulate within these communities (Samie et al., 2020).

Seasonality

Similar to any protozoan parasite *Giardia* is generally found to be more prevalent during the rainy season compared to the drier winter season in most cases. In regions where the infection is endemic, Giardiasis has been observed throughout the year, with slightly higher rates during the pre-monsoon, monsoon, and post-monsoon seasons, particularly from May to October (Tejan et al., 2023). The warm and humid conditions during these periods create favorable conditions for the survival of *Giardia* cysts, and the increased rainfall and flooding contribute to the transmission of infections (Ehsan et al., 2013).

Zoonoses

Research on the infectivity of *Giardia* cysts isolated from both humans and animals has indicated the potential for zoonotic transmission (Monis and Thompson, 2003). The primary zoonotic risk is associated with genotypes of *Giardia* in assemblage A, and to a lesser extent, genotypes in assemblage B (Thompson, 2000). However, when genotypes are determined using multilocus sequence typing, only assemblage A, not assemblage B, seems to have zoonotic potential (Lebbad et al., 2009; Sprong et al., 2009). Various species, including humans, dogs, cats, domestic livestock (such as cattle, sheep, pigs, horses, and goats), and certain species of wildlife are recognized as natural hosts of *G. lamblia* assemblage A (Armson et al., 2009; Yang et al., 2009). In the case of *G. lamblia* assemblage B, natural hosts include humans, dogs, guinea pigs, rabbits, domestic livestock (cattle, sheep, and horses), and certain species of wildlife (Minvielle et al., 2008; Traub et al., 2005; Lebbad et al., 2009). Moreover, *G. lamblia* assemblages A and B have been identified

in marine animals, including dolphins, porpoises, seals (ringed seals), common eiders, and thresher sharks. Although the likelihood of human infection from these animals is minimal, they can contaminate water used by humans for recreational activities (Yang et al., 2010). A study also detected *G. lamblia* assemblage E in human stool samples in Egypt based on the *tpi* gene, which is known to be found in pigs (Foronda et al., 2008).

Detection Method

Detection of giardiasis involves identifying cysts or trophozoites in stool samples through microscopic examination using methods like wet mounts, formalin-ethyl acetate sedimentation or permanent staining with iron hematoxylin or trichrome stains after preserving the stool in various solutions such as polyvinyl alcohol (PVA), 10% formalin, sodium acetate-acetic acid-formalin (SAF), and/or merthiolate-iodine-formalin (MIF). Due to the sporadic shedding of cysts, it is recommended to examine multiple stool specimens over time, typically three serial specimens collected every 2–3 days (Hooshyar et al., 2019; Leung et al., 2019). While microscopy, a cost-effective method, is accessible, it is labor-intensive, subjective, and demands expertise. Although fluorescent antibody staining (or immunofluorescence assay, IFA) enhances sensitivity and specificity, it remains time-consuming and requires a skilled microscopist (Ryan et al., 2017). Additionally, microscopic methods cannot differentiate between species and assemblages as effectively as molecular methods.

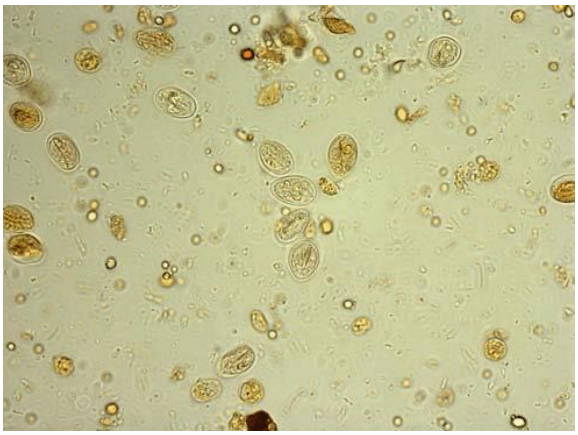


Fig 2.4: Cysts of *G. lamblia* at 400x magnification, unstained wet mount

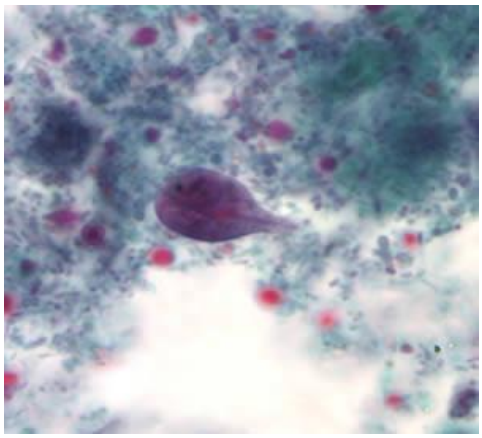


Fig 2.5: *G. lamblia* trophozoite at 400x magnification, trichrome stained

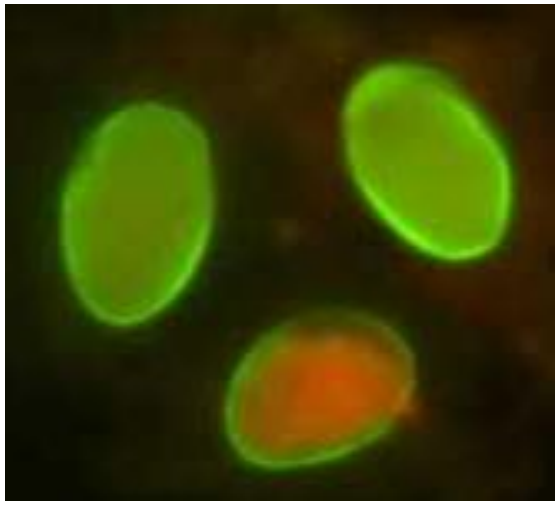


Fig 2.6: Cysts of *G. lamblia* labeled with commercially available immunofluorescent antibodies

Molecular techniques are now extensively employed for detecting *Giardia* in both humans and animals, significantly enhancing our comprehension of transmission patterns (Thompson and Ash, 2016). PCR-based methods, especially, offer speed and objectivity compared to microscopy. They enable species and assemblage determination through DNA sequencing, detection of multiple

targets via multiplexing, and result quantification (Ryan et al., 2017; Xiao and Feng, 2017). Consequently, the adoption of molecular approaches in *Giardia* testing enhances our insight into transmission patterns and the zoonotic potential of isolates. Key genes commonly targeted for *Giardia* identification and molecular characterization include small subunit ribosomal DNA (SSU-rDNA), internal transcribed spacers (ITS1 and ITS2), ß-giardin (*bg*), triosephosphate isomerase (*tpi*), and glutamate dehydrogenase (*gdh*) genes (Ryan and Cacciò, 2013; Thompson and Ash, 2016; Xiao and Feng, 2017). Although SSU-rDNA is a highly conserved multi-copy gene with greater amplification success, it lacks the resolution of other single-copy genes necessary for subtyping (Thompson and Ash, 2016).

Alternatively, rapid fecal antigen detection tests, such as immunochromatographic and enzyme-linked immunosorbent assay (ELISA), have been developed and are widely utilized (Hooshyar et al., 2019; Soares and Tasca, 2016). However, these tests are susceptible to false-positive and false-negative outcomes (Ryan et al., 2017).

Molecular characteristics

Genotyping *G. lamblia* typically involves partially sequencing one or more of the following loci: small subunit ribosomal RNA (SSU rRNA), glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), or β-giardin (*bg*) (Zajaczkowski et al., 2021). Less commonly targeted loci include elongation factor 1-alpha (ef-1) (Traub et al., 2004), GLORF-C4 (C4) (Yong et al., 2002), and the intergenomic rDNA spacer region (IGS) (Lee et al., 2006). Initially, most genotyping studies utilized a single gene for characterizing isolates. However, due to erroneous results, current genotyping methods target more than one locus to identify *G. lamblia* assemblages.

Multilocus PCR is widely acknowledged as the primary method for effective genotyping of *G. lamblia.* Assemblage A, in particular, comprises six distinct subtypes, labeled A1–A6, which are further categorized under three sub-assemblages: AI includes A1 and A5, AII comprises A2, A3, and A4, and AIII consists of A6. The differentiation of these subtypes often depends on detecting a single point mutation, indicating minor genetic variations. However, interpreting genotyping results for assemblage B using this method is complex due to extensive genetic variability and high allelic sequence heterogeneity (ASH) present in this assemblage. While the SSU rRNA gene remains a commonly used option for typing, it is recommended strictly for confirming the presence or absence of *Giardia* DNA in specimens, rather than for subtyping purposes. This is because a major limitation of using any SSU rRNA primer is the low genetic variation between assemblages, making it impossible to differentiate between sub-assemblages. Subtyping G. intestinalis isolates at the level of sub-assemblages is only achievable when utilizing the single-copy genes *tpi, gdh*, or *bg*. Compared to the SSU rRNA gene, the *tpi* and *gdh* markers exhibit the highest discriminatory power, followed by the *bg* gene, indicating their ability to support major assemblage and sub-assemblage typing.

In current genotyping studies, cases of mixed assemblages (A+B) are frequently encountered, presenting challenges in accurately assigning specific genotypes to the isolates. Mixing within assemblages can occur when genetic exchanges happen between different assemblages within a single cyst, leading to the formation of hybrid recombinants. Allelic sequence heterogeneity (ASH) levels are reported to be as low as 0.01–0.03% in assemblage A and 0.4–0.5% in assemblage B, suggesting that *Giardia* may potentially undergo sexual reproduction (Zajaczkowski et al., 2021). Therefore, a novel multi-locus genotyping method is required—one that utilizes polymorphic genetic markers to accurately identify assemblages without concealing true mixed infections.

Treatment

The initial drug discovered and widely utilized for treating giardiasis was quinacrine, serving as the preferred choice for an extended period. With a prolonged half-life ranging from 5 to 14 days, quinacrine is well absorbed through the gastrointestinal tract. Clinical studies have demonstrated cure rates ranging from 77% to 100%, and it has the potential to reduce cyst viability. However, it is characterized by a bitter taste, frequent vomiting, and a risk of inducing toxic psychosis. Moreover, its administration requires three times daily for five to 7 days, posing a challenge for some patients to complete the entire treatment course (Paget et al., 1989).

In the 1960s, Metronidazole emerged as the first 5-nitroimidazole and gained widespread use for the treatment of giardiasis and various infections caused by anaerobic protozoa or bacteria. Metronidazole is typically prescribed for a duration ranging from 5 to 10 days. The drug is transformed into toxic intermediates within anaerobic cells, exerting diverse effects, including DNA reactivity. While Pyruvate-ferredoxin oxidoreductase (PFOR) and thioredoxin reductase (TrxR) have been suggested to contribute to its activity, there are likely other contributing pathways as well. Metronidazole has been associated with potential carcinogenic activity due to its DNA reactivity; however, clinical studies have found little or no correlation. The drug is not teratogenic and has been widely used during pregnancy after the first trimester. Despite never receiving FDA approval for giardiasis, Metronidazole, along with other nitroimidazoles, is now the preferred treatment. It is orally absorbed at 100%, and its absorption from mucosal surfaces, such as the oral, vaginal, and rectal mucosa, is also efficient. A limited study demonstrated the effectiveness of rectally administered metronidazole in treating giardiasis in children. Due to its efficient absorption, levels in the intestinal lumen may be relatively lower compared to less efficiently absorbed drugs, but the impact on clinical activity remains unknown (Adam, 2021).

More recent 5-nitroimidazoles drugs that have been approved are tinidazole and secnidazole. Tinidazole, a metronidazole derivative utilized in Europe since 1969, it did not receive FDA approval until 2004 (Fung and Doan, 2005). Secnidazole gained FDA approval in 2017, although not specifically for treating giardiasis, while ornidazole is still awaiting approval (Ordonez-Mena et al., 2018). These medications boast extended half-lives compared to metronidazole and have all undergone assessment for single-dose administration.

Treatment Refractory Giardiasis

Irrespective of the specific drug and treatment plan employed, achieving a 100% parasitological cure rate in giardiasis cases is uncommon, highlighting the potential for clinical treatment failure. Similar to bacteria, the prolonged use of antimicrobials contributes to the emergence of resistance in parasites. Clinicians have observed instances of treatment-resistant giardiasis for many years. Over the past five decades, susceptibility to metronidazole (MTZ) treatment has transitioned to increasing resistance, and cases of refractory giardiasis have also been documented after the use of all available drugs. A study conducted in Madrid from 1989 to 2004, involving 170 giardiasis cases, revealed that 10 individuals (5.8%) did not respond to one or more initial nitroimidazole (NI)-containing regimens; two of them had IgA deficiency, and one had lung cancer (Lopez-Velez et al., 2010; Muñoz et al., 2013; Requena-Méndez et al., 2017). Subsequently, a small study in Israel identified 12 individuals with NI-refractory giardiasis between 2008 and 2013 (Meltzer et al., 2014). The frequency of NI treatment-refractory cases among giardiasis patients referred to the Hospital of Tropical Diseases in London rose from 15% in 2008 to 45% in 2013. Cases originating from the Indian subcontinent proved particularly challenging, with refractory instances reaching 70% (Nabarro et al., 2015). Likewise, in a retrospective study of 95 Spanish travelers returning from various countries and attending a travel clinic, 21 individuals (22%) exhibited refractory

giardiasis, especially in those returning from Asian countries (Muñoz et al., 2013). In a more recent study involving three specialized Tropical Diseases Units in Barcelona, Spain, a prospective analysis of patients with chronic giardiasis revealed that approximately 20% were unresponsive to treatment with tinidazole or metronidazole (Requena-Méndez et al., 2017).

Prevention and Control

Thorough handwashing plays a crucial role in reducing the risk of *Giardia* infection and preventing its spread, particularly in cases of person-to-person, zoonotic, and foodborne transmission. Individuals at higher risk of infection and transmission, including children and staff in daycares, travelers, animal handlers, veterinarians, petting zoo visitors, and food handlers, need to exercise caution. It is important to exclude symptomatic individuals and those recently recovered, whenever possible, from activities like daycare, swimming, and food handling, as they pose a transmission risk. Public health initiatives and educational programs on personal and food hygiene are essential for increasing awareness and minimizing the transmission risk of giardiasis and other enteric pathogens. In the case of waterborne transmission, employing a multiple barrier approach, such as limiting access to watersheds and reservoirs and utilizing flocculation, filtration, and disinfection, is necessary to minimize the risk (Efstratiou et al., 2017b). While chlorine-treated water still carries a risk due to the resilience of *Giardia* cysts, many filtration methods used in water treatment effectively remove these cysts. Although technologies like ultraviolet light, ozone, and irradiation can inactivate parasites, their cost and limited availability in smaller communities or developing regions make them less common. If drinking water is suspected of *Giardia* cyst contamination, bottled water can be an alternative, and boiling tap water for at least one minute, serves as an emergency measure. Since fresh produce is often consumed raw, implementing effective control measures at the pre-harvest stage, including using treated water, monitoring farm worker health

and hygiene, improving on-farm sanitation, and restricting animal access to crops and surface waters, is crucial (Dixon, 2016; FAO/WHO, 2014). At the food handler and consumer level, emphasizing good personal hygiene, particularly regular handwashing, is vital to reduce the risk of food contamination with protozoan parasites. Travelers to regions with higher giardiasis prevalence should receive instructions through travel clinics and public health messaging to exercise caution in selecting foods and beverages and to maintain good personal hygiene (Leung et al., 2019).

Entamoeba Spp.

Introduction

The *Entamoeba* genus comprises a collection of single-celled, anaerobic, parasitic microorganisms that can be found in humans, nonhuman primates (NHPs), as well as various vertebrate and invertebrate species across the globe. Various species of *Entamoeba* can infect and are typically found in the human intestinal lumen. These include *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni, E. polecki,* and *E. bangladeshi* (Ngobeni et al., 2017). Notably, *E. gingivalis* is primarily located in the human oral cavity but has also been observed in the genitourinary tract of intrauterine contraceptive device users in Egypt (Foda and El-Malky, 2012). In addition, *E. nuttalli*, commonly found in nonhuman primates (NHPs), was identified in a zoo caretaker. Among these infections, pathogenic *E. histolytica* and nonpathogenic *E. dispar* accounted for 81.73% of cases, while the remaining infections were attributed to *E. moshkovskii* (10.22%), *E. coli* (1.98%), *E. hartmanni* (0.96%), *E. polecki* (0.04%), *E. gingivalis* (4.58%), and *E. nuttalli* (0.02%) (Levecke et al., 2015). The overall molecular prevalence of *Entamoeba* infection is 3.55% (3817 out of 107,396 individuals) in humans worldwide. This global prevalence varied, ranging from 1.72% in Oceania to 21.58% in North America. In Europe, cases of *Entamoeba* infection are primarily observed among returning travelers or immigrants (Evangelopoulos et al., 2001; Herbinger et al., 2011; Hove et al., 2009). Previous studies have indicated that travelers face a higher risk of infection with either *E. histolytica* or *E. dispar* when visiting destinations in West Africa, East Africa, South Asia, and Southeast Asia (Herbinger et al., 2011). In certain countries in Asia, Africa, and North America, children and school students appear to be at greater risk of contracting

amebiasis compared to the general population. Similarly, *E. invadens*, found in reptiles, shares a morphological resemblance with *E. histolytica* but is not transmissible to mammals.

Taxonomy

Entamoeba are protozoan parasites known for their pseudopod-forming behavior, belonging to the subphylum Sarcodina within the phylum Amoebozoa. They fall under the class Archamoebae, a group characterized by reduced mitochondria, a feature common among various anaerobic eukaryotes and the family Entamoebidae (Tovar et al., 1999). Alongside *Entamoeba*, this group encompasses other intestinal inhabitants such as *Endolimax* and *Iodamoeba*, which share a resemblance with *Entamoeba*, possibly attributed to convergent evolution. The Archamoebae also includes free-living amoebo-flagellates found in the genus Mastigamoeba and related genera (Stensvold et al., 2012). The genus *Entamoeba* comprises at least seven distinct species (*Enatomeba histolytica, Entamoeba coli, Entamoeba hartmanni, Entamoeba polecki, Entamoeba dispar, Entamoeba moshkovskii, and Entamoeba bangladeshi*), which primarily inhabit the human intestine. Additionally, there is one species, *Entamoeba gingivalis*, found in the oral cavity.

Amoebiasis, a neglected tropical disease caused by *Entamoeba* sp., stands as one of the leading causes of death globally among parasitic diseases, profoundly impacting millions of individuals. The status of various *Entamoeba* spp. as commensals or pathogens has been a persistent subject of debate, fueled by sporadic reports of infections attributed to *Entamoeba dispar* (*E. dispar*) and *Entamoeba moshkovskii* (*E. moshkovskii*), which were initially considered commensal. Among the different species of *Entamoeba* found in humans, *Entamoeba histolytica* (*E. histolytica*) is recognized as pathogenic from the outset. It is well-established that *E. histolytica* has the potential to cause both invasive and non-invasive infections, whereas *E. dispar* and *E. moshkovskii* are

associated exclusively with non-invasive infections. This review will specifically focus on these three clinically relevant species concerning enteric infection.

Kingdom: Protista

Sub-Kingdom: Protozoa

Phylum: Amoebazoa

Class: Archamoebae

Order: Mastigamoebida

Family: Entamoebidae

Genus: *Entamoeba*

Entamoeba histolytica, a member of the genus *Entamoeba*, is capable of infecting humans and is considered pathogenic in comparison to other species such as *Entamoeba moshkovskii*, a species that has been more recently associated with human infections, and the human commensal *Entamoeba dispar*. Notably, *Entamoeba moshkovskii* exhibits greater diversity than *E. histolytica* across the genome. The former is approximately 200 times more diverse than the latter, with the *E. moshkovskii* strains having a most recent common ancestor nearly 500 times more ancient than the tested *E. histolytica* strains. The results of a four-haplotype test suggest that the tested *E. moshkovskii* strains are distinct and should be classified as a species complex.

Clinical features: Amebiasis

Amebiasis, which is among the most prevalent parasitic diseases, significantly contributes to the burden of diarrhea, particularly in underdeveloped regions with inadequate sanitation practices

(Costa, 2018). Amebiasis, amebic dysentery, is a gastrointestinal infection solely caused by *Entamoeba histolytica* (*E. histolytica)*, with the ability to invade the lining of the intestinal tract and potentially spread to other organs, primarily the liver. Notably, the National Institute of Allergy and Infectious Diseases (NIAID) classifies *E. histolytica* as a category B priority biodefense pathogen (Shirley et al., 2018). In animal models, amoebic dysentery can be induced by a relatively small number of cysts, ranging from 1 to 100, which is comparable to the infective dose of the highly contagious *Shigella* sp. The pathogenic amoeba, *E. histolytica*, displays variable virulence. Infection can manifest as subclinical or clinical disease. It resides within the large intestine and cecum, and its presence may result in no apparent clinical symptoms. Alternatively, it may invade the intestinal mucosa, giving rise to mild to severe ulcerative hemorrhagic colitis. In cases of heightened virulence, trophozoites have the capability to enter capillaries and migrate to other organs, including the brain, liver, and lungs. In contrast, *E. dispar*, which is genetically distinct, is a noninvasive and nonpathogenic amoeba, despite sharing morphological similarities with *E. histolytica* (Roberts J, 2022).

Clinical manifestations of amebiasis vary depending on the affected organ, manifesting as either intestinal or extraintestinal symptoms. In the case of invasive intestinal amebiasis, there are four primary clinical forms, all of which tend to be acute: dysentery or bloody diarrhea, fulminating colitis, amebic appendicitis, and ameboma of the colon. Dysentery and diarrheic syndromes collectively account for around 90% of cases of invasive intestinal amebiasis. Patients with dysentery typically experience an average of three to five mucosanguineous evacuations per day, accompanied by moderate colic pain preceding discharge and rectal tenesmus. In the case of bloody diarrhea, evacuations are infrequent, but the stools consist of liquid fecal material stained with blood. Although moderate colic pain may be present, rectal tenesmus is not. Generally, fever

and systemic symptoms are absent. These syndromes represent the classic presentation of ambulatory dysentery and can be readily distinguished from bacterial infections, where patients often exhibit systemic signs such as fever, chills, headache, malaise, anorexia, nausea, vomiting, cramping abdominal pain, and tenesmus (Martínez-Palomo and Espinosa, 1998).

E. histolytica can infect virtually any organ in the body, but the most common form of extraintestinal amebiasis is the amebic liver abscess. This condition arises when trophozoites migrate from the colon to the liver through the portal circulation. It is more prevalent in adults, being 10 times more common in adults than in children and three times more frequent in males compared to females (Sepúlveda and Treviño-García, 1986). Typically, the onset of amebic liver abscess is abrupt, marked by pain in the right hypochondrium radiating towards the right shoulder and scapular area. The pain tends to intensify with deep breathing, coughing, and when pressure is applied while walking on the right foot. In cases where the abscess is localized to the right lobe, symptoms may include an irritative cough, sometimes accompanied by productive coughing, and a pleuritic chest pain. Abscesses in the upper left lobe can lead to epigastric discomfort, sometimes causing dyspnea and spreading pain to the base of the neck and both shoulders. Fever, typically ranging from 38 to 40°C, is present in approximately 85 to 90% of patients with amebic liver abscess. Chills and profuse sweating, particularly in the afternoon and at night, are common. Other symptoms encompass anorexia, nausea, vomiting, diarrhea (with or without blood), and dysentery. On physical examination, a key indicator of amebic liver abscess is painful hepatomegaly. Digital pressure and fist percussion often provoke intense pain in the liver region. The liver itself feels soft and smooth, in contrast to the rough, hard, and irregular texture observed in patients with cirrhosis and hepatocarcinoma. Jaundice is seen in 8% of patients who respond well to treatment. Severe jaundice may indicate the presence of multiple abscesses. Diarrhea or dysentery is observed

in less than one-third of patients. Complications of amebic liver abscess can include perforation into the pericardial space, pleura, or peritoneal cavity (Espinosa-Cantellano and Martínez-Palomo, 2000).

Amebiasis has become a significant public health concern, responsible for approximately 70 thousand deaths each year. It ranks as the fourth leading cause of death attributed to a protozoan infection, following malaria, Chagas' disease, and leishmaniasis. Furthermore, it is the third most common cause of morbidity within this category, trailing behind malaria and trichomoniasis, as per recent estimates from the World Health Organization (WHO). Invasive amebiasis caused by *E. histolytica* is more prevalent in developing countries. Within regions of endemic infection, several factors, including lack of awareness, poverty, overcrowding, inadequate and contaminated water sources, and poor sanitation, create conditions conducive to direct fecal-oral transmission of amoebas from one person to another. Diagnosing amebic liver abscess can be challenging at times. In regions where the infection is endemic or in cases with a history of travel to such areas, clinicians should consider amebic abscess in patients who present with spiking fever, weight loss, and abdominal pain in the upper right quadrant or epigastrium, along with tenderness in the liver area. Additional indicators include leukocytosis, elevated alkaline phosphatase levels, and an elevated right diaphragm, which can suggest the presence of a hepatic abscess. Confirmation of the diagnosis is typically achieved through ultrasonography or computed tomography (CT) scans. CT scans, in particular, offer the highest precision in identifying hepatic abscesses, especially when they are small. Moreover, with the administration of intravenous contrast agents, CT scans prove invaluable in the differential diagnosis of other focal liver lesions (Sepúlveda and Treviño-García, 1986).

Transmission

Upon ingestion via fecally contaminated food or water, the infective *E. histolytica* cysts undergo passage through the stomach and excyst in the terminal ileum. Here, they mature into trophozoites that colonize the large intestine by adhering to colonic mucins and feed on bacteria from the intestinal microbiota (Faust and Guillen, 2012). Initially, commensal trophozoites become invasive, leading to the destruction of the muco-epithelial barrier. This process induces the overproduction of mucus, causes the death of host cells, and triggers inflammation, ultimately resulting in dysentery. Trophozoite populations can attain high densities and aggregate, potentially leading to a transition from exponential growth to encystation (Faust and Guillen, 2012). In some instances, the parasites may traverse the portal vein, reaching the liver and causing the primary extraintestinal infection known as amoebic liver abscess. Additionally, in immunocompromised patients, *E. histolytica* can travel to the lungs and brain (Carrero et al., 2020). To perpetuate the life cycle, *E. histolytica* cysts are excreted in stool and contribute to further fecal-oral transmission. Human-to-human transmission has also been reported through oral-genital and oral-anal contact, particularly among individuals with poor personal hygiene and in certain populations, such as homosexuals (Li et al., 2021).

Epidemiology

The estimated global molecular prevalence of *Entamoeba* spp. infection in humans is 3.55%. *Entamoeba* species that are commonly found in the human intestinal lumen include *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni, E. polecki, and E. bangladeshi* (Ngobeni et al., 2017). Notably, pathogenic *E. histolytica* and nonpathogenic *E. dispar* accounted for the majority, representing 81.73% of these infections, followed by *E. moshkovskii* at 10.22% (Cui et al., 2019).

In 1903, Schaudinn was the first to describe and distinguish *E. histolytica* from non-pathogenic *Entamoeba* species. *E. histolytica*, the third highest cause of death from parasite diseases, remains a major global health concern. Each year, around 500 million individuals worldwide experience the effects of amoebiasis, with approximately 10% being infected by *E. histolytica*, while the remaining cases are caused by non-pathogenic *Entamoeba* species. According to the Global Burden of Disease Study, amoebiasis is accountable for over 100,000 annual fatalities across the globe and results in 2.2 million disability-adjusted life years (DALYs). The infection is widespread in tropical and subtropical areas, particularly in Central and South America, Asia, and Africa. Among the most affected nations, Bangladesh, India, Brazil, Colombia, Mexico, and China are prominent. This disease primarily affects travelers, immigrants, individuals in the LGBTQ+ community, and prisoners (Nasrallah et al., 2022). *E. histolytica*, specifically in South Africa, exhibited a notable prevalence rate of 12.4%. In Kenya, the estimated prevalence of intestinal amoebiasis caused by *E. histolytica* was 58.3%, while in Rwanda, it was 54.5%, and in Uganda, it was approximately 19.93%. Egypt reported a high rate of asymptomatic *E. histolytica* infections detected in stool samples i.e., 21%. The prevalence of extraintestinal amoebiasis attributed to *E. histolytica*, determined through serology, ranged from 15% to 70%. In Abidjan, 92% of treated patients experienced sequellae, and late diagnosis resulted in a mortality rate of up to 15%. In Cameroon, the most frequent form of extraintestinal amoebiasis was amoebic liver abscess (ALA), with 97% of 188 patients presenting with this condition (Nasrallah et al., 2022).

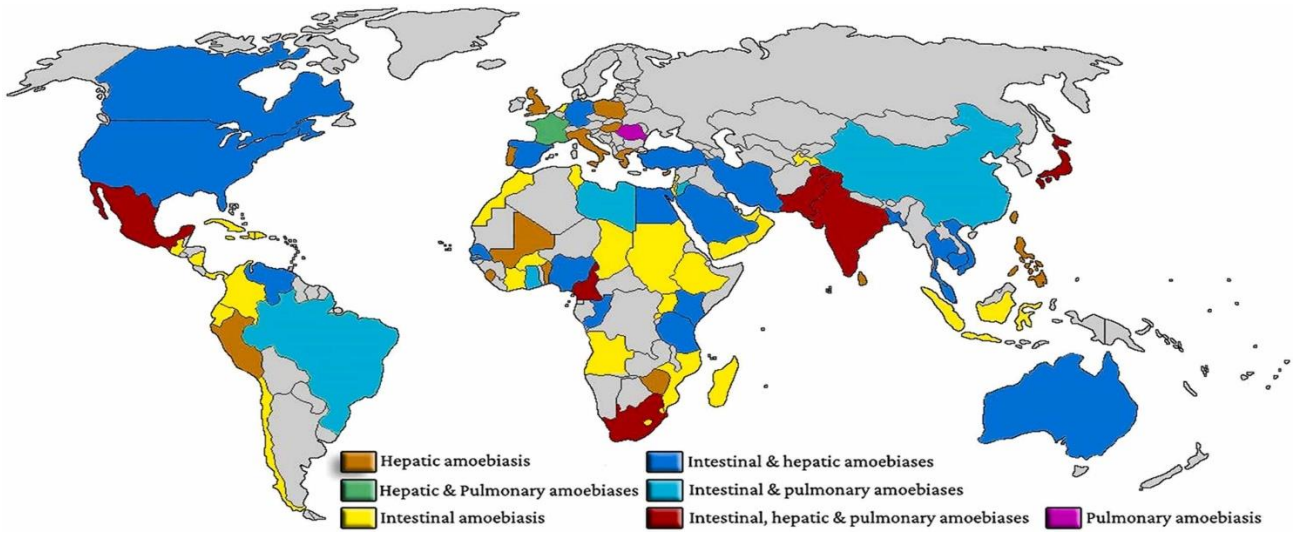


Fig 2.7: Global distribution of endemic foci of intestinal and extraintestinal amoebiasis

conditions prevail. In the Indian subcontinent, the prevalence of intestinal amoebiasis attributed to *E. histolytica* was approximately 11.7% in 2011 (Samie et al., 2012). Bangladesh, part of this subcontinent, also exhibited a high prevalence of intestinal amoebiasis, with a prevalence rate of 4.2% reported among children living in urban slums of Dhaka, where 11% of Bangladeshi children experienced diarrhea in their first year of life (Mondal et al., 2012). The United Arab Emirates reported a prevalence rate of 30% for *E. histolytica*, and in Iran, the prevalence was reported to be approximately 3.45% (Stauffer et al., 2006). In the Philippines, hepatic abscess was the most common extraintestinal complication of amoebiasis, with 83 cases of hepatic infection reported in Manila between 1967 and 1975. Vietnam experienced remarkably high incidence rates of amoebic liver abscess, with annual values of approximately 21 cases per 100,000 inhabitants (Blessmann et al., 2002). *E. histolytica* seropositivity was found to be 11% across seven provinces of China. Moreover, in the Gaza Strip, the average annual incidence rate of both intestinal and extraintestinal amoebiasis from 2008 to 2017 was 358.8 cases per 100,000 inhabitants. The rise in immigration

and travel from endemic regions has given rise to the emergence of amoebiasis in developed countries in North America. During the period from 1990 to 2007, 134 deaths were recorded, with a higher prevalence in men (Gunther et al., 2011). In 2007 alone, California reported 411 cases of intestinal amoebiasis attributed to *E. histolytica*, with an estimated prevalence of nearly four percent. In Mexico, the annual incidence rate for intestinal amoebiasis ranged from one to five thousand cases per 100,000 inhabitants between 1995 and 2000. This rate increased to 1128.8–615.85 per 100,000 inhabitants from 2002 to 2006 (Ximénez et al., 2009). The National Epidemiological Surveillance System in Mexico documented over 8.8 million cases of amoebiasis between 2000 and 2010 (Bottazzi et al., 2011). Amoebiasis has become a public health concern in Europe, primarily introduced from other endemic regions. The incidence of intestinal amoebiasis in central Europe, such as the Czech Republic, has generally been low; however, there is a possibility of underreporting as the disease often eludes diagnosis. In Spain, advancements in water infrastructures over the last century have facilitated the eradication of endemic intestinal amoebiasis. Interestingly, 23 cases of amoebic liver abscess were identified in Spanish patients who had never left the Iberian Peninsula. In the context of extraintestinal amoebiasis, France reported 152 cases of hepatic amoebiasis with liver abscess. These cases were documented over 15 years since 1984 in two military hospitals. The second-largest series of imported cases was reported in the early 2000s in the Paris area, with travelers and foreign-born individuals being the most affected (Nasrallah et al., 2022).

The significance of the amphizoic amoeba *Entamoeba moshkovskii* is growing in the investigation of amoebiasis, particularly in certain developing countries. Initially described as a distinct species by Tshalaia in 1941, *E. moshkovskii* was thought to be a free-living environmental strain, commonly found in anoxic sediments to brackish coastal pools. In 1961, an *E. histolytica*-like

strain was isolated from a resident of Laredo, Texas, experiencing symptoms like diarrhea, weight loss, and epigastric pain. Named *E. histolytica* Laredo strain, it shared many biological characteristics with *E. moshkovskii*. Molecular studies later revealed that the *E. histolytica* Laredo strain is indeed *E. moshkovskii*, marking the first human isolate of this species. Human colonization by *E. moshkovskii* has been reported in various countries, including the United States, Italy, Iran, Turkey, Bangladesh, India (specifically Pondicherry), Kenya, Australia, Indonesia, Colombia, Malaysia, Tunisia, Tanzania, and Brazil. In Bangladesh, this species has been identified as the likely pathogen in individuals with gastrointestinal clinical manifestations, including dysentery. A study from Eastern India found that 3.12% of individuals were infected with *E. moshkovskii* (Sardar et al., 2023). In Colombia, the first study reporting human infections with *E. moshkovskii* found it in 25.4% of cases (Lopez et al., 2015). These studies indicate that *E. moshkovskii* is among the contributing factors to the occurrence of acute diarrhea in humans.

Host Range and Zoonotic Potential

Humans serve as the primary hosts for *E. histolytica, E. moshkovskii*, and *E. dispar*. Cysts of these species originating from humans have also been identified in nonhuman primates (NHPs) (Li et al., 2021). Additionally, these *Entamoeba* species have been reported in both domestic and wild animals, encompassing pigs, cattle, sheep, goats, horses, deer, rodents, reptiles, and elephants (Cui et al., 2019). Infections with *E. histolytica* have been documented in NHPs globally, including instances in Belgium (Levecke et al., 2010), the Netherlands (Verweij et al., 2003), and China (Jiang et al., 2008). Previous studies have identified *E. histolytica* in diarrheic captive and wild lemurs, suggesting symptomatic *E. histolytica* infections in these lemurs (Berrilli et al., 2011; Ragazzo et al., 2018). Moreover, a study in Malaysia reported the occurrence of *E. histolytica* in wild rats (Lau et al., 2014). The zoonotic potential of *E. moshkovskii* is not extensively explored,

and reports are limited. Two recent studies, one from Malaysia reporting *E. moshkovskii* in dogs and another from India identifying pigs as potential animal reservoirs of *E. moshkovskii*, contribute to our understanding of its distribution (Ngui et al., 2020; Sardar et al., 2022).

Life Cycle of *E. histolytica*/ *E. moshkovskii*/ *E. dispar*

The life cycle of *E. histolytica/ E. moshkovskii/ E. dispar* begins with major routes of transmission, including the consumption of contaminated water and food or through direct fecal–oral contact. Additionally, transmission of *E. histolytica* can occur sexually via the anal-oral route. The infective cyst form demonstrates robustness, resisting chlorination, gastric acidity, and desiccation, allowing it to persist in moist environments for several weeks. The mobile stage of *Entamoeba*, known as the trophozoite, resides within the large intestine lumen, where it undergoes multiplication and transformation into the cyst—a resilient form pivotal for infection transmission. Cysts are expelled in feces and can be ingested by a new host through contaminated food or water. Upon reaching the terminal ileum, the parasite undergoes excystation, with each emerging quadrinucleate trophozoite generating eight uninucleated trophozoites. The trophozoites of *E. histolytica* have the potential to invade the colonic mucosa, leading to dysentery. Furthermore, through dissemination via the bloodstream, they may result in extraintestinal lesions, predominantly in the form of liver abscesses.

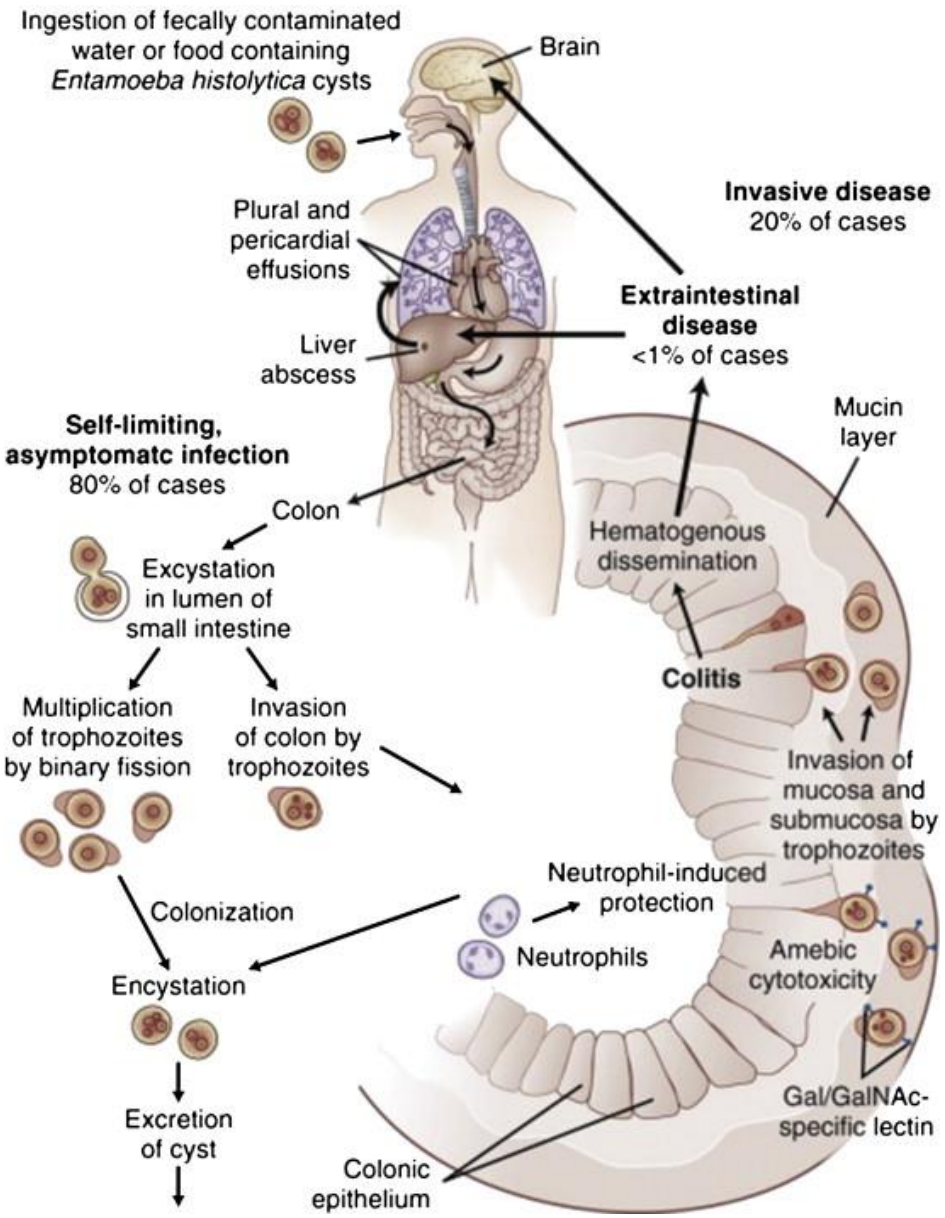


Fig 2.8: Life cycle of *Entamoeba histolytica*

Upon ingestion, the cyst form of *E. histolytica/ E. moshkovskii/ E. dispar* traverses the stomach and enters the small bowel, where excystation takes place. During this process, the cyst undergoes nuclear and subsequent cytoplasmic division, giving rise to eight trophozoites, which then migrate to the large bowel. The multiplication of trophozoites happens through binary fission. Frequently,

the newly formed trophozoites aggregate in the intestinal mucin layer and create new cysts, leading to a self-limited and asymptomatic infection. Trophozoites may colonize the bowel lumen as commensal flora. Alternatively, *E. histolytica* trophozoite invasion of the colonic epithelium can occur, resulting in extensive inflammation and the destruction of the bowel wall. The factors influencing invasion as opposed to colonization or asymptomatic illness remain unknown. Potential contributors include variations in invasiveness among different genotypes of *E. histolytica*, host genetic differences, variations in the host gut microbiome, the nutritional status of the host, and the immunocompetence of the host. Following invasion, amoebae can disseminate to the liver through hematogenous spread involving the hepatic portal circulation. Hematogenous dissemination and direct extension from a liver abscess can also lead to rare extra-abdominal involvement of the CNS, pulmonary, or cardiac organs (Royer and Petri, 2014). Trophozoites undergo multiplication through binary fission, giving rise to cysts, as depicted in the image, and both stages are excreted in the feces. Cysts can endure for days to weeks in the external environment, remaining infectious due to the protective nature of their walls. In contrast, trophozoites passed in the stool are swiftly eliminated once outside the body. If ingested, they would not survive exposure to the gastric environment (https://www.cdc.gov/dpdx/amebiasis/index.html).

Morphology

E. dispar, designated as a distinct species in 1993, is morphologically identical to *E. histolytica* and is recognized as its closest described relative (Weedall and Hall, 2011). Under bright-field microscopy, *E. histolytica/E. dispar* cysts appear spherical and typically measure 12 to 15 µm (with a range of 10 to 20 µm). A mature cyst possesses four nuclei, while an immature cyst may contain only 1 to 3 nuclei. The peripheral chromatin is fine, uniform, and evenly distributed. Occasionally,

elongated chromatoid bodies with bluntly rounded ends may be observed. The presence of diffuse or absent glycogen characterizes mature cysts, whereas immature cysts exhibit clumped glycogen. Trophozoites in trichrome-stained smears generally measure 15 to 20 µm (with a range of 10 to 60 µm). Morphological features of trophozoites include one nucleus with evenly arranged chromatin on the nuclear membrane and a small, centrally located karyosome. The cytoplasm appears finely granular, with a few ingested bacteria or debris possibly present. The diagnostic feature for identifying *E. histolytica* is the presence of red blood cells within the cytoplasm of trophozoites. While the ingestion of red blood cells is not always common, the absence of this characteristic should lead to reporting as *E. histolytica/ E. dispar* (https://www.cdc.gov/dpdx/resources/pdf/benchaids/entamoeba_benchaid.pdf).

The morphology of *E. moshkovskii* under light microscopy is also indistinguishable from the two *Entamoeba* species, *E. histolytica* and *E. dispar*. In its trophozoite form, this amoeba has a diameter ranging from 9 to 25 µm, with a more common size falling between 11 µm and 13 µm. The cystic form exhibits a mean size that varies between (10.3 ± 0.1) µm and (11.82 ± 0.13) µm, depending on the strain. Iron hematoxylin staining is preferred for studying its structure, revealing 1–4 nuclei (1.5–2 µm in diameter), although forms with more nuclei are observed. There is considerable variability in nuclei morphology, including chromatoid bodies ranging from 3 µm to 7 µm per cyst. These bodies have an elongated shape with round edges and tend to occupy a central position in mature cysts.

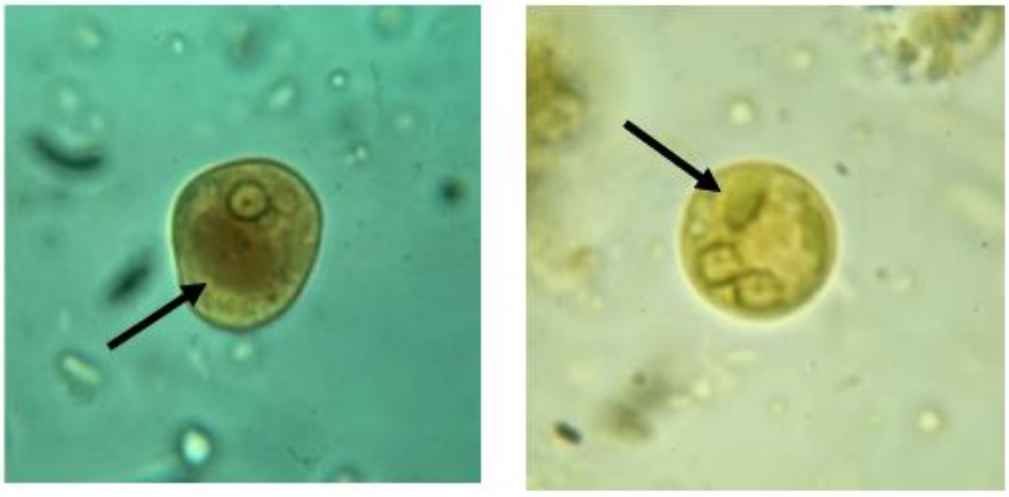


Fig 2.9: Cysts of *Entamoeba* spp. wet mount (at 400x magnification)

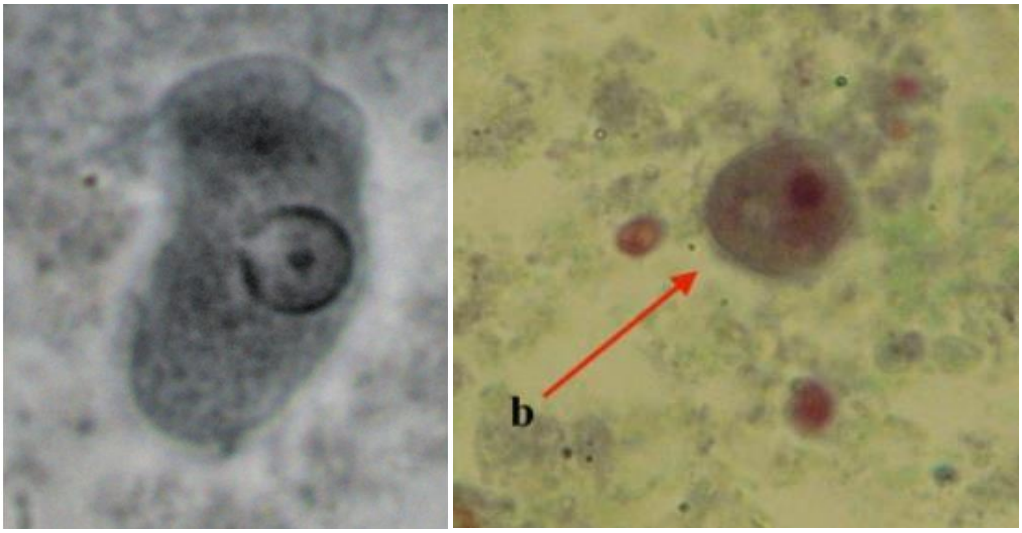


Fig 2.10: Trophozoite of *E. moshkovskii* iron hematoxylin stain (at 400x magnification)

Transmission

Amoebiasis primarily affects young adults and is uncommon in children below the age of 5. Amoebic dysentery is particularly rare in children under 2 years, as dysentery in this age group is

more commonly attributed to *Shigella* spp. Outbreaks have been documented following contamination of water supplies, given the resistance of cysts to chlorine. Humans, often asymptomatic carriers passing cysts in feces, serve as the reservoir for the parasite. Amoebiasis can be transmitted through: 1) ingestion of food or water contaminated with amoebic cysts, 2) indirect hand contamination from surfaces contaminated with cysts, and 3) oral–anal sexual contact with a chronically ill or asymptomatic carrier. *E. histolytica* can be transmitted through heterosexual activity, as well as male and female homosexual activity. Patients with amebiasis should receive counseling about potential sexual transmission. Cases remain infectious as long as cysts are present in the feces, and in some instances, cyst excretion may persist for years. All non-immune individuals are susceptible to infection, whereas those with *E. dispar* do not develop symptoms. Re-infection is possible but rare (https://www.health.vic.gov.au/infectious-diseases/amoebiasis). Infection with *E. moshkovskii* may lead to acute diarrhea in both humans and pigs, as suggested by some reports (Sardar et al., 2023; Sardar et al., 2021).

Seasonality

A potential factor influencing the transmission of *E. histolytica* is seasonality. *E. histolytica/E. dispar* tends to exhibit higher prevalence in the rainy season when water is more abundant and weather is moist. These protozoan parasites are typically found at higher levels in summer and the rainy season, with the lowest prevalence in winter. There is often an association between diarrheal disease and season, as observed in various studies (Haque et al., 2006). Research has indicated marked seasonality in *E. histolytica* infection in humans, with peaks during the wet season (Mukherjee et al., 2010). However, conflicting findings exist, with some studies reporting no clear relationship between *E. histolytica* infection and season. In the case of *E. moshkovskii*, a specific seasonal distribution has been identified for its infection. The prevalence of *E. moshkovskii*

parasites tends to increase during the post-fall (Sep-Nov) season and in the summer season (April-June) each year (Sardar et al., 2023).

Detection Method

Microscopy has traditionally been the predominant method for identifying *Entamoeba* (Fotedar et al., 2007). Historically, the epidemiology of amebiasis relied heavily on microscopy as the primary diagnostic tool, which lacked the ability to differentiate true infections caused by *E. histolytica* from those caused by non-pathogenic *Entamoeba* spp. i.e., *E. dispar, E. moshkoskii* (Turkeltaub et al., 2015). Microscopic identification of cysts and trophozoites in stool can be achieved using wet mounts with or without iodine stain, as well as permanently stained preparations (e.g., trichrome) of fresh stool or concentrates from fresh stool. Cysts of *E. histolytica/E. moshkovskii/E. dispar* exhibit a spherical shape and typically measure 12 to 15 μm, with a range of 10 to 20 μm. A mature cyst contains four nuclei, while an immature cyst may have only 1 to 3 nuclei. Trophozoites in trichrome-stained smears generally measure 15 to 20 μm, with a range of 10 to 60 μm. Morphological features of trophozoites include one nucleus with evenly arranged chromatin on the nuclear membrane and a small, centrally located karyosome (https://www.cdc.gov/dpdx/amebiasis/index.html). In recent times, molecular tools have gained increasing prominence for the identification, taxonomy, epidemiology, and clinical significance of *Entamoeba* spp. The small subunit rRNA (SSU rDNA) gene has been widely utilized for phylogenetic analysis among eukaryotic organisms and for detecting *Entamoeba* spp. in stool samples. Various molecular diagnostic tests are now available for the diagnosis of intestinal amoebiasis, including conventional PCR, nested PCR, real-time PCR, multiplex PCR, and loop-mediated isothermal amplification assay (LAMP) (Khademerfan et al., 2019). Conventional PCR and nested PCR targets various genes, including SSU rDNA, DNA with highly repetitive

sequences, the hemolysin gene HLY6, cysteine proteinase, the serine-rich *E. histolytica* SREHP gene, the actin gene, and tandem repeats in extrachromosomal circular DNA, to discriminate between *Entamoeba* species. (Freitas et al., 2004; Zindrou et al., 2001). Laboratory diagnosis of *Entamoeba* spp. has extensively embraced real-time PCR assays owing to their heightened sensitivity, elimination of post-PCR manipulation, and decreased risk of contamination (Fotedar et al., 2007). The advent of multiplex real-time PCR further enables swift and simultaneous identification, genotyping, and quantification of multiple DNA targets within a single reaction (Hamzah et al., 2010). In resource-limited regions, the LAMP assay, recognized for its high sensitivity, specificity, rapidity, and simplicity, emerges as a favorable molecular diagnostic option.

Commercially available enzyme immunoassay (EIA) kits for the detection of *E. histolytica* antibodies and antigens are accessible in the United States. Antibody detection proves most beneficial in patients with extraintestinal disease, such as amebic liver abscess, where organisms are not typically identified through stool examination. However, antibody detection has limited diagnostic value in patients from highly endemic areas who are likely to have prior exposure and seroconversion. It may be more useful in patients from regions where pathogenic *Entamoeba* spp. are rare (https://www.cdc.gov/dpdx/amebiasis/index.html).

Molecular Characteristics

Entamoeba displays a notable degree of genomic plasticity and instability. The utilization of molecular tools for studying the molecular epidemiology of *E. histolytica* and *E. dispar* infections in endemic regions provides a valuable means to discern the distribution of genetic variants of these two species across different geographic areas. The potential benefit of this knowledge lies in predicting potential morbidity surges in at-risk communities by understanding the geographic

distribution of virulent genotypes of *E. histolytica*. Unfortunately, to date, there is a lack of convincing evidence establishing a direct relationship between genotypes and the specific amoebic outcomes in infected hosts such as amebic colitis, amebic liver abscess, amebic brain abcess or asymptomatic intestinal infection. Establishing any association between *E. histolytica* and clinical outcomes of infections necessitates reliable diagnostic assays for genotyping the parasite. Consequently, various diagnostic techniques have been developed over the years, with polymerase chain reaction (PCR)-based approaches proving highly sensitive and discriminatory in this regard.

Advancements in molecular detection techniques, have been made using various polymorphic molecular targets. Notably, polymorphic genetic loci such as short tandem repeats (STRs) in the intergenic region related to tRNA genes, including DA-H5/H3 (Hsp 1–2), NK-H5/H3, StgaD-H5/H3, SQ5/SQ-H3 for *E. histolytica*, and DA-D5/D3 (Dsp 1–2), NK-D5/D3, StgaD-D5/D3, SQ-D5/SQ-D3 for *E. dispar*, have been extensively studied. Genome rearrangement, linked to tissue invasion and organ tropism, has been proposed as a potential explanation for the distinct tRNA short tandem repeat (STR) genotypes observed in liver abscess and stool-derived parasites originating from the same infected individual. While both species of *Entamoeba* exhibit a high degree of polymorphism, it is evident that *E. histolytica* displays a lower level of polymorphism compared to *E. dispar* (Rojas et al., 2016). However, the distinctive pattern of variable short tandem repeats observed in *E. histolytica* is absent in *E. moshkovskii*, where these regions exhibit variability without the characteristic repetition (Weedall and Hall, 2011). Additionally, apparent variations in lipophosphopeptidoglycan and lipophosphoglycan-like molecules have been confirmed across avirulent and virulent *E. histolytica* strains. DNA typing of polymorphic genetic loci, such as the serine-rich *E. histolytica* protein (SREHP), which is a highly immunogenic surface protein involved in *E. histolytica* phagocytosis, has revealed significant genetic polymorphism in

both coding and non-coding loci among different *E. histolytica* isolates (Kareem et al., 2023). Comprehending the factors influencing the infection outcome and the broader nature of amoebic virulence serves as a significant driver for extensive *Entamoeba* research.

Treatment

Amebiasis is typically managed with amebicides, and the choice of medication depends on the severity of the infection (Martínez-Castillo et al., 2018). These drugs are categorized as luminal amebicides (such as paromomycin, diloxanide furoate, iodoquinol, and nitazoxanide) and tissue amebicides (chloroquine, emetine, tinidazole, and metronidazole (MTZ)), based on their respective sites of action. Metronidazole (MTZ) stands out as the most recommended and widely used drug for treating invasive amebiasis (Gonzales et al., 2019; Shirley et al., 2018). Despite its widespread use, amebicidal concentrations of MTZ have been found to induce parasite resistance under laboratory conditions. Studies have indicated that this resistance may be linked to an increased expression of iron-containing superoxide dismutase and peroxiredoxin. Additionally, partial resistance to MTZ has been reported in certain clinical strains of *E. histolytica*, suggesting the emergence of MTZ-resistant strains. In Egypt, two studies investigating the treatment efficacy of nitazoxanide in *E. histolytica*-induced diarrhea suggested its potential effectiveness (Li et al., 2021) Further research is underway to explore new drugs with distinct targets and modes of action from those of MTZ (Ansari et al., 2020; Nagaraja and Ankri, 2019).

Prevention and Control Measures

Amoebiasis can be effectively prevented and controlled through a combination of specific and non-specific measures. Specific measures primarily involve the use of vaccines targeted against amoebiasis, particularly for *E. histolytica*. These vaccines are known for being cost-effective, safe,

and providing long-lasting protection with minimal side effects. Vaccination using both native and recombinant forms of the parasite Gal/GalNAc-lectin has demonstrated efficacy in protecting animals against intestinal amebiasis and amebic liver abscess. Protection against amebic liver abscesses can also be achieved by targeting other *E. histolytica* components, including the serine-rich protein and the 29-kDa-reductase antigen (Li et al., 2021). However, considering their relevance to the pathogenesis of the disease, the Gal/GalNAc lectin from *E. histolytica* appears to be the most promising target in the diagnosis, vaccination, and treatment of these infections. Clinical trials will be necessary to validate its efficacy in humans (Guzmán-Téllez et al., 2020; Quach et al., 2014). Non-specific measures, such as sanitation and personal hygiene, take precedence in the prevention and control of amoebiasis. Specifically, the focus should be on improving water supply, excreta disposal, and food safety (Lejeune et al., 2009). In endemic areas, ensuring an adequate supply of water for handwashing and food preparation may be more critical than the water's quality alone (Samie et al., 2020). The implementation of these preventive measures at both individual and community levels is essential for effectively preventing and controlling amoebiasis.

Cryptosporidium spp.

Introduction

Cryptosporidium spp. are widely known as significant contributors to diarrheal outbreaks of parasitic origin, associated with contaminated water and food sources on a global scale. *Cryptosporidium* spp. can infect both domestic animals, livestock, and humans, leading to gastrointestinal illness known as cryptosporidiosis. These protozoan parasites are consistently reported as significant environmental pollutants in various countries, regardless of their distinct climatic, socioeconomic, and demographic characteristics (El-Alfy and Nishikawa., 2020). It has been recognized as the sixth most significant food-borne parasites worldwide. Similar to other enteric parasites, these microorganisms are excreted in the feces of infected hosts and can transmit to new hosts through fecal-contaminated soil, water, animal feed, and food. The genus *Cryptosporidium* was initially identified as a human pathogen in 1976 (White, 2010). The transmission pathways for human infections encompass person-to-person transmission, transmission through contaminated food, transmission via contaminated water and zoonotic transmission. The primary clinical manifestation of human cryptosporidiosis typically includes copious watery diarrhea accompanied by abdominal pain, low-grade fever, nausea, vomiting, and weight loss. In immunocompetent individuals, the condition often presents as asymptomatic, mild, or self-limiting, whereas in immunosuppressed individuals, such as in HIV patients, it can be severe and potentially fatal. *Cryptosporidium* spp. infections can also lead to fatalities in various mammalian animals and result in chronic illness in reptiles (Plutzer et al., 2018). The parasite, previously considered rare and host-specific, is now recognized as ubiquitous and capable of infecting multiple hosts (Current et al., 1991). Presently, there are nearly 30 species of

Cryptosporidium and over 40 genotypes that have been identified to infect a wide range of mammals, birds, reptiles, and fish (Xiao et al. 2004; Ryan et al. 2014). Their classification is based on various factors including oocyst size and morphology, life cycle characteristics, and host specificity (Khan et al. 2018). Among these, *C. hominis* and *C. parvum* are the most common culprits for causing cryptosporidiosis in humans, accounting for approximately 90% of infections. Nonetheless, numerous other species like *C. meleagridis, C. ubiquitum, C. felis*, and *C. canis* have also been documented as causes of the disease. *Cryptosporidium*, therefore pose a significant public health and environmental concern, with human epidemics often poorly understood, particularly in non-industrialized regions. On a global scale, cryptosporidiosis stands as a serious challenge, manifesting as acute, chronic, or persistent diarrhea in children. This condition leads to growth stunting, physical deterioration, impaired cognitive function, and, in severe cases, can result in fatalities. Accurate diagnosis plays a pivotal role in clinical management, and a comprehensive understanding of the epidemiology of human cryptosporidiosis is deemed essential for devising crucial interventions for this public health issues.

Taxonomy

Cryptosporidium spp. is an intracellular, protozoan parasite classified within the Apicomplexa phylum. In 1907, Ernest Edward Tyzzer initially described a coccidian parasite under the genus name *Cryptosporidium*. He documented the type species, *C. muris*, which he found within the gastric glands of a tame, laboratory mice. The parasites were found to adhere to the epithelial cell surface via knob-like projections. Later, he also identified a closely related smaller species in the small intestines of rabbits and named it *C. parvum* (Fayer and Xiao, 2008; Hassan et al., 2020). This parasitic protozoa with anthropo-zoonotic potential belong to the phylum Apicomplexa, class *Sporozoasida*, subclass *Coccidiasina*, order *Eucoccidiida*, suborder *Eimeriina*, family

Cryptosporidiidae (Ghazy et al., 2015.). More recently, the genus *Cryptosporidium* has been categorized within the subclass *Cryptogregaria*, which is a part of the class *Gregarinomorphea* (Ryan et al., 2016).

Kingdom: Protista

Sub-Kingdom: Protozoa

Phylum: Apicomplexa

Class: Gregarinomorphea

Sub-Class: Cryptogregaria

Order: Eucoccidia

Family: Cryptosporidiidae

Genus: *Cryptosporidium*

Before 1971, the genus *Cryptosporidium* was limited to three recognized species: *Cryptosporidium muris*, *Cryptosporidium parvum*, and *Cryptosporidium meleagridis*. In the early 1970s, reports emerged linking *Cryptosporidium* to bovine diarrhea, although specific species were not identified at the time. In 1971, a new species, *Cryptosporidium wrairi*, was identified in guinea pigs, increasing the total number of valid species to four by that year (Ryan et al., 2021). Initially, it was believed that each *Cryptosporidium* species had a narrow host range, infecting only a single host or closely related species. For example, *Cryptosporidium canis* was primarily associated with dogs (Khan et al., 2018). Throughout that decade, cryptosporidiosis in various animals, including sheep, pigs, horses, turkeys, rabbits, monkeys, snakes, cattle, and guinea pigs had been reported. During this period, several new species were described, including *Cryptosporidium agni* in sheep,

Cryptosporidium bovis in calves, *Cryptosporidium anserinum* in geese, *Cryptosporidium cuniculus* in rabbits, and *Cryptosporidium garnhami* and *Cryptosporidium enteriditis* in humans (Ryan et al., 2021). However, it is now evident that *Cryptosporidium* has been found in more than 150 distinct host species. Some *Cryptosporidium* species, such as the cervine genotype *Cryptosporidium ubiquitum*, have a broad host range, including domestic and wild ruminants, rodents, woodchucks, deer, raccoons, and humans. The first two reports of cryptosporidiosis in immunocompromised humans and its association with diarrhea were published in 1970s. *C. parvum* infects a wide range of hosts, including humans, ruminants, and cattle under 2 months old (Khan et al., 2018). Until the late 1990s, it was widely believed that most intestinal *Cryptosporidium* species were *C. parvum* genotypes. For instance, *Cryptosporidium hominis* was referred to as *C. parvum* genotype I or *C. parvum* human genotype, and *Cryptosporidium canis* was identified as *C. parvum* canine genotype. There are now a minimum of 44 validated *Cryptosporidium* species, with several originally identified as genotypes and later officially recognized as distinct species. These include six avian *Cryptosporidium* species (*C. avium, C. baileyi, C. galli, C. meleagridis, C. ornithophilus and C. proventriculi*), four piscine species (*C. abrahamseni, C. bollandi, C. huwi and Cryptosporidium molnari*), one amphibian species (*C. fragile*), four reptile species (*C. serpentis, C. varanii, C. testudines, and C. ducismarci*), and 29 mammalian species. In 2004, a new piscine species- *Cryptosporidium scophthalmi*, was identified in cultured turbot (*Scophthalmus maximus*), based on morphological and biological characteristics. (Ryan et al., 2021). *Cryptosporidium viatorum*, initially considered a minor pathogen in humans, was subsequently discovered to infect various animals, including rat species and chipmunks (Chen et al., 2019; Koehler et al., 2018; Sardar et al., 2020). With ongoing genetic and biological research, it is likely that new *Cryptosporidium* species will be identified, necessitating further

epidemiological studies to differentiate between zoonotic (animal-to-human) and anthroponotic (inter-human or human-to-animal) transmission.

Life Cycle

The life cycles of all *Cryptosporidium* species share a common pattern. Typically, the infection commences with the consumption of sporulated oocysts, each containing four sporozoites (Khan et al. 2018). The median infectious dose is approximately 312 oocysts (DuPont et al. 1995), but even as few as 10 oocysts can initiate infection in humans (Okhuysen et al. 1999). Within each oocyst, the motile sporozoites are liberated within the gastrointestinal tract through a break in the oocyst wall via excystation (Bouzid et al. 2013). This excystation process can be triggered by various factors following ingestion, including gastric pH, body temperature, pancreatic enzymes, and bile salts (Hijjawi et al. 2001). Once released, the sporozoites releases proteins to facilitate attachment and subsequent invasion of the host cells (Wanyiri 2006). Generally, sporozoites have a crescent-shaped structure, featuring an apical complex housing micronemes, a single rhoptry, and dense granules (O'Hara & Chen 2011). Micronemes release their contents, which facilitate adherence to host cells through the secretion of thrombospondin-related protein. The rhoptry may aid in host cell invasion and parasitophorous vacuole membrane (PVM) formation (Spano et al. 1998; Soldati et al. 2001). Dense granules likely discharge their contents after PVM formation, potentially modifying this structure to facilitate host cell invasion (Tetley et al. 1998).

With the assistance of the rhoptry and micronemes, the parasite invades the host cell, including the cell membrane, enveloping the parasite in the PVM (Bouzid et al. 2013). The PVM is notable for remaining extra-cytoplasmic but is considered intracellular, maintaining its position within the host-derived PVM atop epithelial cells (Tzipori & Ward 2002). Within the PVM, the parasite is shielded from the harsh gut environment and gains energy and nutrients from the host cell through

a specialized and highly invaginated membrane known as the feeder organelle. This organelle forms between the parasite and the host cytoplasm during internalization (Tzipori and Ward, 2002).

Within the PVM, each sporozoite undergoes transformation into a spherical trophozoite. These trophozoites reproduce asexually through a process called merogony, forming Type 1 meronts that contain eight merozoites. Once mature, they invade neighboring intestinal epithelial cells. Type I meronts have the option to develop into more Type I meronts, thereby intensifying the infection rapidly. Alternatively, they can also progress into Type II meronts, which contain four merozoites (Bouzid et al. 2013). When released, these Type II merozoites attach to other epithelial cells in the intestinal tract. Instead of forming additional meronts, Type II merozoites initiate a process called gametogony, marking the completion of the sexual development phase (Göbel & Brändler 1982). Each merozoite generates either male or female equivalent sexual reproductive stages, referred to as microgamonts or macrogamonts, respectively (Göbel & Brändler 1982). Microgamonts undergo nuclear division to produce up to 16 microgametes, which, upon release from the PVM, fertilize a unicellular macrogametocyte, developed from a macrogamont, forming a zygote (Current & Reese 1986). The diploid zygote then undergoes a process akin to meiosis, known as sporogony, resulting in the formation of four haploid sporozoites within a sporulated oocyst (Current & Reese 1986).

The resultant oocysts come in two forms: thick-walled and thin-walled, each containing four sporozoites. Thick-walled oocysts are resilient to environmental conditions and are excreted into the environment with the host's feces, facilitating the transmission of infection from one host to another. In contrast, thin-walled oocysts remain in the intestine, capable of auto-infecting the same host. Autoinfection transpires when sporozoites are released from the thin-walled oocyst, initiating the cycle anew (Ryan et al., 2021).

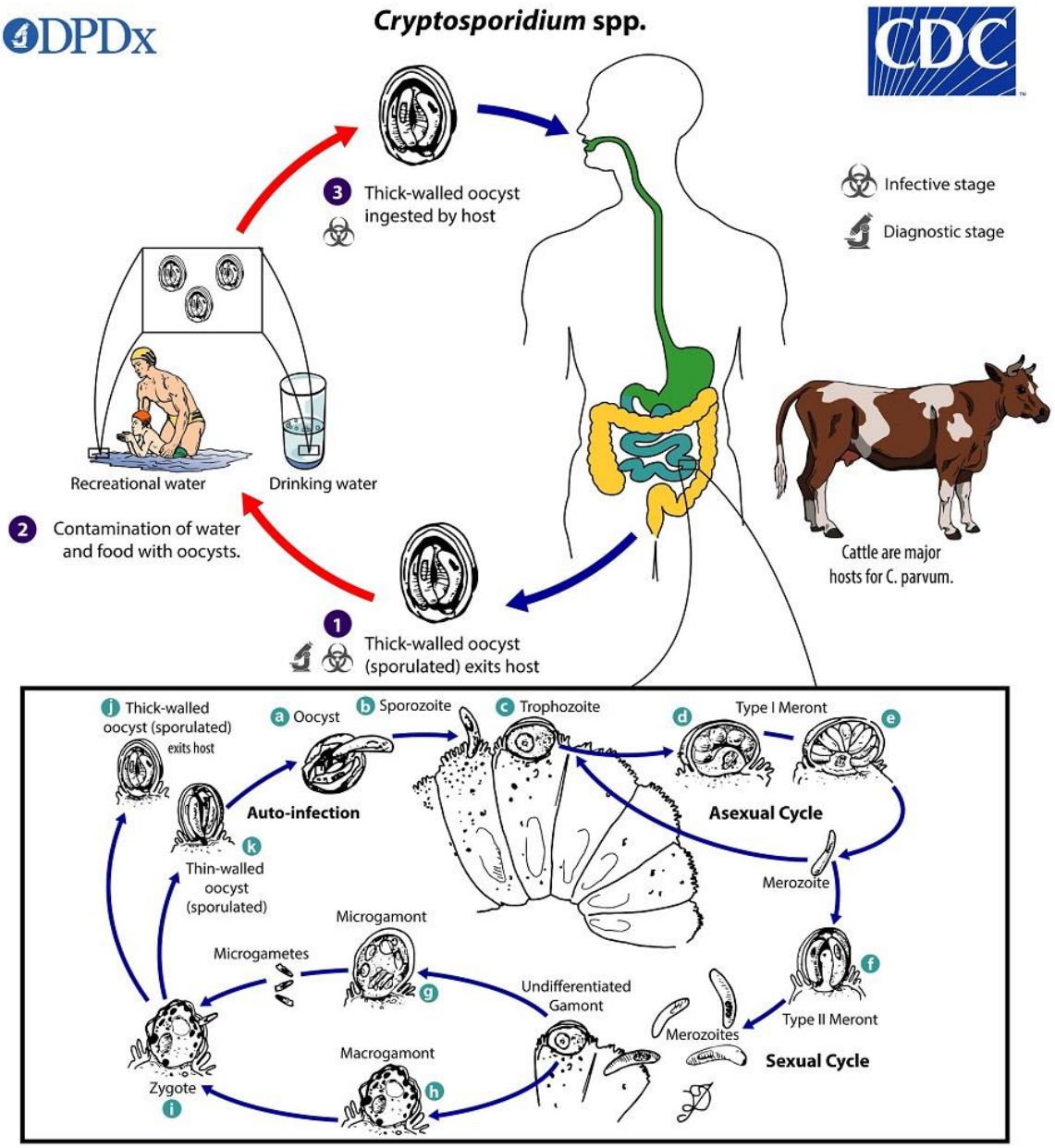


Fig 2.11: Transmission cycle of *Cryptosporidium* spp.

Morphology

The parasite undergoes six distinct morphological stages during its life cycle. The endogenous developmental stages (trophozoite, meronts, gamonts) manifest as small, basophilic structures (3-6µm) either affixed to the luminal surface of host epithelial cells or inside the enterocytes.

In contrast, the exogenous oocysts take on the appearance of ovoid, phase-bright bodies (measuring 5-7 x 4-6µm) housing four sporozoites and a residual body. The oocyst is the diagnostic form excreted in host feces. It is the smallest group of coccidian known to cause infection. Oocysts are colorless, spherical to oval. They are acid-fast and surrounded by a 50nm thin cyst wall, featuring an electroluscent middle zone encased by two electron-dense layers. Each oocyst contains up to four slender, fusiform sporozoites. These sporozoites are slender, crescent-shaped, and measure 1.5 µm- 1.75 µm in diameter. They remain parallel within the oocyst and are released only after partial oocyst digestion. The anterior end of the sporozoite is rounded, and it invades enterocytes in the host (Eman et al., 2021).

Inside enterocytes, the sporozoites differentiate into intracellular trophozoites. Trophozoites are typically round or oval and measuring 2 µm - 2.5 µm in diameter. Each trophozoite contains a large nucleus, with or without a conspicuous nucleolus. These trophozoites multiply asexually by to produce two types of meronts: Type I and Type II. Meronts are crescent-shaped and measure 1 µm - 5 µm in diameter, featuring rounded anterior and posterior ends. They, in turn, produce Type I and Type II merozoites that resemble sporozoites and amplify asexual infectious cycles. Some of the Type II merozoites invade new host cells and initiate sexual replication. Within the host cells, some differentiate into male forms, known as microgamonts. Each microgamont produces 16 separate rod-like, non-flagellated microgametes measuring 1.4×0.5 µm in size. Some of the Type II merozoites differentiate into a spherical or oval structure, typically 4 - 6 µm in diameter,

housing a large central nucleus called macrogamont. These macrogamonts are fertilized by microgametes to form oocysts or zygotes (Humagain, 2021).

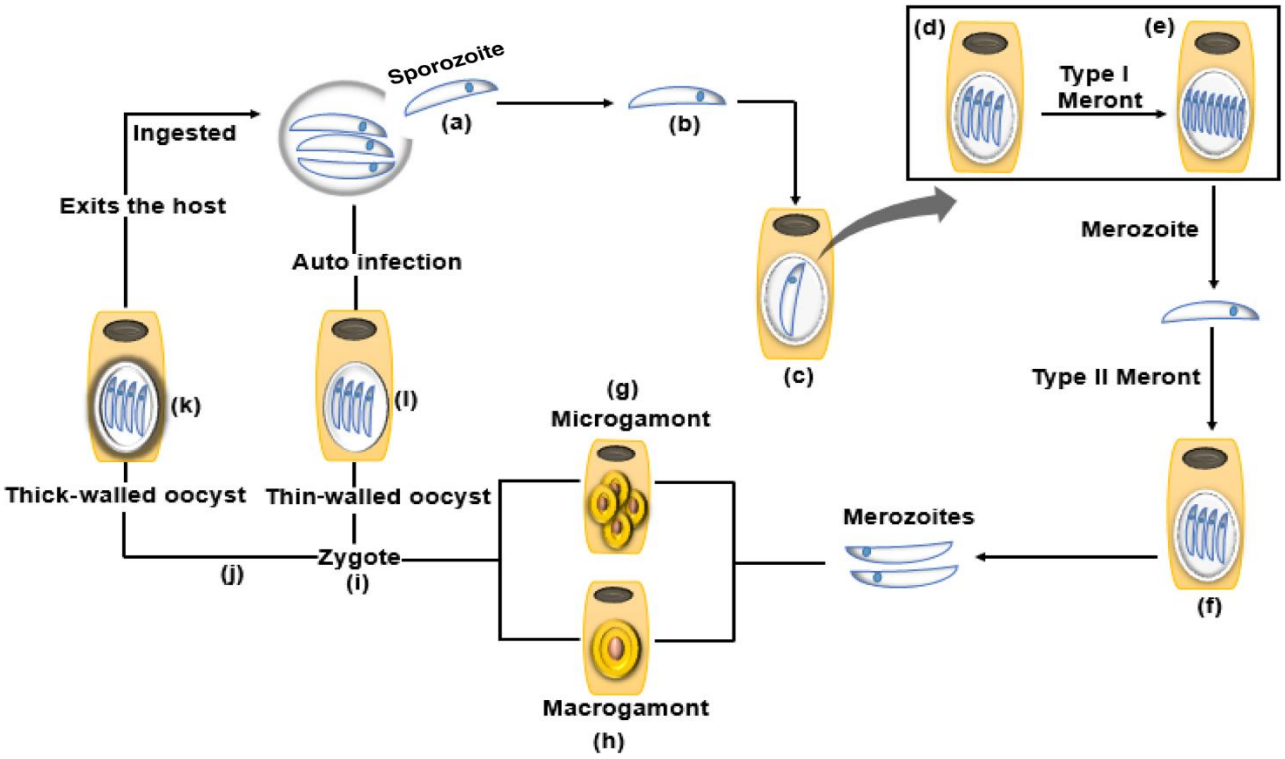


Fig 2.12: Six distinct morphological stages of *Cryptosporidium* spp. during its life cycle.

Human Cryptosporidiosis: Clinical Features

Cryptosporidiosis has emerged as one of the most prevalent human enteric infections of parasitic origin. Initially, it went largely unnoticed before the onset of the acquired immune deficiency syndrome (AIDS) pandemic. Typically contracted by children, adults are also susceptible. The disease gained notoriety due to its severe consequences in immunosuppressed individuals (Griffiths, 1998). While human illness was initially attributed to a single species, molecular studies have revealed that various species contribute to human cryptosporidiosis. Among the more

common ones are *Cryptosporidium hominis*, with humans as the sole natural host, and *Cryptosporidium parvum*, capable of infecting both bovines and humans (Ayuo, 2009). Initially considered a commensal organism, *Cryptosporidium* gained attention when it was linked to turkey enteritis in 1955. The heightened interest in veterinary investigations started in 1971, with the recognition of its significance in causing bovine diarrhea. The first report of *Cryptosporidium* as a human pathogen came in 1976, involving an immunocompetent 3-year-old girl with acute enterocolitis who spontaneously recovered (Crawford and Vermund, 1988). Cryptosporidiosis stands out as one of the most prevalent causes of diarrhea in humans, with its peak incidence observed in children under two years old (Ayuo, 2009). The condition is characterized by symptoms such as watery diarrhea, abdominal pain, nausea, vomiting, and low-grade fever asting 5-10 days, with the possibility of persistence for 2-4 weeks (Cacciò and Chalmers, 2016). In individuals with AIDS and very low CD4 cell counts, the disease may progress to profuse, cholera-like diarrhea, leading to complications such as volume depletion and malabsorption. AIDS patients may also experience biliary tract involvement, which can manifest as acalculous cholecystitis, sclerosing cholangitis, papillary stenosis, or pancreatitis (Ayuo, 2009). Volunteer transmission studies indicate that immunocompetent adults can be infected with as few as 30 oocysts, although reinfection after one year typically requires about ten times that amount (DuPont et al., 1995; Griffiths, 1998). Another form i.e., fulminant cryptosporidiosis, characterized by a dramatic cholera-like illness and exceptionally high mortality, is primarily observed in individuals with AIDS or those undergoing immunosuppression due to chemotherapy.

Chronic cryptosporidial disease is most prevalent among individuals with AIDS or malnutrition. The survival rate for individuals with HIV infection and chronic cryptosporidiosis is notably shorter compared to those with transient or asymptomatic disease, with a significant difference

observed (20 weeks versus 36 weeks). The reduced survival in the former group may be attributed to factors such as wasting and dehydration, as evidenced by lower weights (64.7 kg versus 59.3 kg). The majority of affected individuals found relief through antiviral therapy and/or opiates to manage their diarrhea. Among the subjects with both cryptosporidiosis and AIDS, 27 out of 38 experienced chronic diarrhoea, leading to a mean survival time of only 11.5 weeks. In contrast, those who experienced remission had a significantly longer mean survival time of 66 weeks (Griffith 1998).

The presence of asymptomatic carriers may play a crucial role in the endemic transmission of *Cryptosporidium*. In industrialized nations, reports often indicate very low carriage rates of *Cryptosporidium* in individuals without gastrointestinal symptoms. Asymptomatic individuals who are nonetheless infected tend to have a low parasite load (Crawford and Vermund, 1988).

In a study conducted in a New York City day-care center involving asymptomatic subjects, 6 out of 31 children and 1 out of 14 staff members tested positive for a few or rare *Cryptosporidium* oocysts. Asymptomatic carriage rates may be higher in areas characterized by low socioeconomic development and increased exposure.

In immunocompromised patients, *Cryptosporidium* infection has been documented to affect extraintestinal sites, including the esophagus, bile ducts, gall bladder, pancreatic duct and pancreas, liver, and lungs. The concept of asymptomatic biliary carriage of *C. parvum* potentially serving as a reservoir for sporadic transmission exists, but convincing or confirming evidence for this reservoir is currently lacking. For instance, Roberts et al. (1989) examined 169 immunocompetent adults undergoing routine upper gastrointestinal endoscopy in New York, USA. They found that 12.7% had *Cryptosporidium* in duodenal aspirates, although none had positive duodenal biopsies.

This suggested infection in the biliary tract of these individuals. Half of those with positive aspirates had oocysts in stool samples, yet none experienced diarrhea (Griffith, 1998).

In the early stages of infection, *Cryptosporidium* induces blunting of the intestinal villus and hypertrophy of the intestinal crypts. Animal studies indicate that secretory (crypt) capacity remains intact while absorptive function decreases. A neutrophilic infiltrate typically appears within 36-48 hours. In piglets, localized prostaglandins and tumor necrosis factor inhibit Na+ absorption, leading to net chloride secretion. Overt disruption of the intestinal mucosa by cryptosporidiosis is uncommon, and cases of pneumatosis cystoides intestinalis, indicating mucosal breakdown, are rare. Malabsorption of nutrients occurs, accompanied by decreased levels of intestinal brush-border digestive enzymes such as lactase and alkaline phosphatase. Vitamin B12 and D-xylose absorption negatively correlate with the intensity of infection, with intestinal injury proportional to the number of infecting organisms. Vitamin A malabsorption has been documented in calves. In persistent cryptosporidiosis in humans, hepatobiliary or pancreatic duct infection may develop. Acute infection in various animal models typically remains confined to the intestinal tract, while chronic infection models often involve the biliary system. Biliary carriage acts as a reservoir for intestinal relapse in individuals with AIDS, even post-treatment with luminal agents like paromomycin, and poses its own risks. Sclerosing cholangitis with papillary stenosis has been increasingly reported in people with AIDS and biliary cryptosporidiosis. This process is characterized by right upper quadrant pain, cholestasis, and other signs of cholangitis. Sphincterotomy can ameliorate the disease.

Respiratory infection is common but often asymptomatic. In childhood cryptosporidiosis, cough is frequent, reported in one-fifth to one-third of normal children. Pulmonary symptoms are about three times more frequent in children admitted to the hospital with cryptosporidial diarrhea than

in those with other intestinal pathogens. It has been suggested that transient respiratory cryptosporidiosis is common in immunocompetent children. In individuals with AIDS, respiratory infection is marked by severe persistent cough and dyspnea. Respiratory cryptosporidiosis is likely prevalent, remains largely undefined as a contributing factor in transmission, and poses a potential fatality risk in immunocompromised individuals (Griffiths, 1998).

Transmission

Both waterborne and person-to-person transmissions are reported, as well as animal-to-animal, animal-to-human, and environmental transmissions. Water contamination, particularly from cattle and other animals, plays a crucial role in zoonotic transmission. Water draining from fields with cattle feces can contaminate water supplies, leading to human outbreaks (Griffiths, 1998). Contamination of water supplies by human sewage or feces remains a persistent concern. Outbreaks can result from plumbing defects and fecally contaminated freshwater swimming pools, despite the routine use of chlorinating agents (MacKenzie et al., 1995a). Day-care center epidemics are frequent and often underdiagnosed. Prospective studies have revealed that asymptomatic childhood carriage of *C. parvum* is common, and unsuspected child-to-child transmission may be a significant factor in endemic disease (Pettoello-Mantovani et al., 1995). Outbreaks tend to occur in day-care centers, custodial institutions, and closed populations like family units (Keusch et al., 1995). Hospital employees and patients face a special risk of acquiring cryptosporidiosis, especially as AIDS patients in hospitals may excrete large numbers of oocysts. Uncooked sausage and contaminated raw milk can also serve as potential vehicles for the transmission of cryptosporidiosis. Several large studies have highlighted the role of sexual transmission. In a prospective cohort study of 6548 people with AIDS (Pedersen et al., 1996), homosexual men were more likely to develop cryptosporidiosis (4.1%) than intravenous drug users (IVDUs). A study

found that intestinal parasitosis, including cryptosporidiosis, in adults with HIV infection was related to anal-penile sex (Esfandiari et al., 1995). These studies strongly suggest that cryptosporidiosis can be sexually acquired. Peridomestic animals, including pets, serve as reservoirs of infection. Over 40 mammalian species may harbor *C. parvum*. Cryptosporidiosis can be acquired after visiting farms, and dairy farmers are particularly at risk. Numerous studies confirm that *Cryptosporidium* spp. can be transmitted to humans from farm animals (Das et al., 2019; Pumipuntu et al., 2018; Guo et al., 2022).

Host Range and Zoonotic Potential

The three most common species of *Cryptosporidium* in humans are *C. parvum, C. hominis*, and *C. meleagridis*. However, *C. parvum* and *C. hominis* are responsible for over 90% of cryptosporidiosis cases. Since the 1980s, cattle have been recognized as a significant source of zoonotic cryptosporidiosis. Contact with infected calves has been implicated in several small cryptosporidiosis outbreaks among veterinary students, research technicians, and children attending agricultural camps and fairs (Chalmers et al., 2005b; Kiang et al., 2006). *C. parvum*, often referred to as the bovine genotype, is known to primarily infect ruminants and humans, although occasional natural infections have been observed in other animals such as mice and raccoon dogs. While cattle have been considered major hosts for *C. parvum*, preweaned calves are more frequently infected, with postweaned calves commonly hosting *C. bovis* and the deer-like genotype. Lambs can also be naturally infected with *C. parvum*, and direct transmission from lambs to children has been confirmed in at least one small cryptosporidiosis outbreak through subtyping (Chalmers et al., 2005b). In numerous accounts of *C. parvum* infection in both humans and animals, the clinical presentation is described as gastroenteritis (Anderson, 1998).

Initially thought to infect only humans, *C. hominis* has been identified in various production animals globally, including cattle, sheep, goats, horses, donkeys, and Bactrian camels (Widmer et al., 2020). The first documented natural bovine infection by *C. hominis* occurred in clinically ill cattle during a survey of cryptosporidiosis in livestock in Scotland, UK (Smith et al., 2005). *C. hominis* has sporadically been reported at low frequency rates in cattle herds across different continents, including Australia, China, France, Kenya, New Zealand, and Uganda. Occasional infections have also been reported in ovine and caprine animals, with the first description of a naturally occurring *C. hominis* infection in sheep reported from Australia (Ryan et al., 2005). Moreover, molecular detection has identified *C. hominis* oocysts in fecal samples from various animals, including migratory birds, ungulates, marsupials, marine mammals, mesocarnivores, frugivorous bats, and rodents. It's important to note that these detections may not necessarily represent true infections (Widmer et al., 2020).

There is another *Cryptosporidium* species, namely *C. viatorum* subtype XVaA3h, known to infect humans. The *C. viatorum* XVa subtype family exhibits a wide host range, infecting not only humans but also various wild animals such as rats, chipmunks, and swamp rats (Sardar et al., 2020; Chen et al., 2019; Koehler et al., 2018).

Epidemiology

The majority of cases of cryptosporidiosis worldwide occur in children. The elevated occurrence of cryptosporidiosis among children under the age of 5 is likely due to heightened exposure to the parasite resulting from inadequate hygiene practices and underdeveloped immunity. It is crucial to recognize that children infected with the parasite are likely to transmit it to the parents. In fact, there is a secondary, smaller peak in prevalence observed in adults aged between 30 to 40 years,

particularly among women, with *C. hominis* being the primary species implicated (Caccio and Chalmers, 2016).

The prevalence of cryptosporidiosis in cases of diarrhea varies, with lower rates observed in cooler, more developed countries (0.1-2% overall, potentially double in children) and higher rates ranging from 0.5-10% in warmer, less developed nations (Griffiths, 1998). In developing nations, the majority of individuals contract *Cryptosporidium* during their childhood. Stool examinations and serological studies have provided evidence of elevated infection rates in Africa, the Middle East, Latin America and South Asia. Globally, approximately 13% of submitted stool samples for parasitological analysis in developing countries show the presence of *Cryptosporidium* oocysts. However, among AIDS patients experiencing diarrhea, the incidence of cryptosporidiosis has been identified to reach as high as 72% (Ayuo, 2009). *Cryptosporidium* proved to be the predominant enteric pathogen detected in the feces of malnourished children (average age of 12 months) experiencing diarrhea in Kingston, Jamaica. Among malnourished children, 24% tested positive for *Cryptosporidium*, in contrast to 3.5% among well-nourished children (Crawford, 1988). In southern India, *Cryptosporidium* was identified in 13.1% of patients under 4 years old experiencing acute diarrhea, and in 9.8% of healthy controls. Among children younger than 6 months, *Cryptosporidium* was actually more prevalent in the control group (21.8%) than in the case group (16.8%). Notably, only in children aged 12 to 18 months was *Cryptosporidium* significantly more frequent among those with acute diarrhea (13.5%) compared to controls (6.1%). In Liberia, *Cryptosporidium* was detected in the stool samples of 8.4% of children experiencing diarrhea, 5.9% of matched controls, and 8.6% of household contacts of children with *Cryptosporidium*. The prevalence of *Cryptosporidium* in children with diarrhea did not show a

significant difference compared to the other groups. Among the *Cryptosporidium*-positive children, 31% were asymptomatic carriers (Crawford, 1988).

In the past decade, instances of cryptosporidiosis outbreaks have been identified in numerous developed nations, linked to factors such as the consumption of food and water, engagement in recreational activities (especially in swimming pools), contact with animals, outdoor pursuits, and the transmission of the parasite through person-to-person contact, both in households and institutions. In the years 2010–2011, Sweden experienced the two most significant outbreaks ever recorded in Europe, collectively impacting an estimated 47,000 individuals. Of notable significance was another outbreak in May 2012, spanning across England and Scotland and involving over 300 reported cases (Caccio and Chalmers, 2016). The etiology of chronic diarrhea in individuals infected with Human Immunodeficiency Virus (HIV) or suffering from AIDS is primarily attributed to *Cryptosporidium* spp. In a comparative study of children with and without HIV infection, both experiencing persistent diarrhea a study in Uganda demonstrated that 73.6% of HIV-positive individuals had *Cryptosporidium,* whereas only 5.9% of those without HIV were affected. In a case-control study involving 103 HIV-infected outpatients in Venezuela, *Cryptosporidium* was found in 13.6% of cases. Additionally, in Nairobi, Kenya, among 75 consecutive in-patients with HIV and diarrhea, 52% were identified with pathogens, with *Cryptosporidium* spp. being the most common, accounting for 17% (Ayuo, 2009). While the protozoan parasite Cryptosporidium spp. was acknowledged as a human pathogen in 1970s, its significance as a causative agent of diarrheal disease gained widespread recognition only after a significant outbreak in 1993. This outbreak, affecting more than 400,000 people and resulting in 69 deaths in Milwaukee, Wisconsin, US, highlighted the substantial impact of *Cryptosporidium* as

a diarrheal disease-causing pathogen. Subsequently, *Cryptosporidium* has been acknowledged as the most frequently identified pathogen in waterborne outbreaks in the United States.

Recent surveys conducted by the Global Burden of Disease (GBD) indicate that *Cryptosporidium* is accountable for over 44.8 million instances of diarrhea and 48,000 deaths annually worldwide. Findings from the Global Enteric Multicenter Study (GEMS) underscore that *Cryptosporidium* is linked to a more than 2-fold increased risk of death among 12- to 23-month-olds with moderate-to-severe diarrhea. Despite this well-documented and substantial burden, the broader global health community tends to under-recognize the impact of cryptosporidiosis. Notably, Cryptosporidiosis is not categorized as a neglected tropical disease (NTD) by the World Health Organization, and it is not included on the list of tropical diseases eligible for a priority review voucher (PRV) from the US Food and Drug Administration (FDA). Researchers worldwide are urging the FDA to promptly approve a recently submitted petition to designate cryptosporidiosis as a tropical disease, thereby providing incentives for the advancement of promising drug candidates (Choy & Huston, 2020).

Detection Method

Most tests for detecting *Cryptosporidium* involve the direct microscopic examination of the parasite in tissues or fecal specimens. In 1983, Ma and Soave proposed a three-step approach for the detection of *Cryptosporidium*: 1. An iodine wet mount of fecal material, where oocysts remain unstained while yeasts take on a brown stain. 2. Utilization of a modified Kinyoun acid-fast (cold method) as the permanent record. 3. Application of Sheather's sugar cover-slip flotation to concentrate oocysts. Modified acid-fast staining or Kinyoun Staining of stool is a commonly employed laboratory test due to its cost-effectiveness and straightforward methodology. In this method, oocysts appear as red-stained rounds against a blue-green background. However, staining methods have shown relatively low sensitivity, ranging from 33.3% to 41% with stool specimens.

To significantly enhance sensitivity, examination of prepared slides under UV light with a rhodamine filter (540-560 nm) can be employed (Ayuo, 2009). It is important to note that in any population of oocysts, there will be some variation in staining. Young oocysts attached to intestinal microvilli may be less acid-fast, and these may be shed into the stool in severe infections. Some oocysts may appear unstained if the preparation is over-decolorized with 10% H_2SO_4, as may happen with a very thick smear, or if there are degenerate, older oocysts that fail to retain the carbol fuchsin stain (Crawford and Vermund, 1988). In bright-field microscopy employing differential interference contrast (DIC), oocysts are observed as small, round structures measuring 4 to 6µm, resembling yeasts. These oocysts typically exhibit distinct oocyst walls and can stain from light pink to bright red. However, staining may exhibit variability. Notably, infections in the resolution phase may display colorless oocyst "ghosts." Mature oocysts may also contain discernible sporozoites, with up to four being present (https://www.cdc.gov/dpdx/resources/pdf/benchaids/crypto_benchaid).

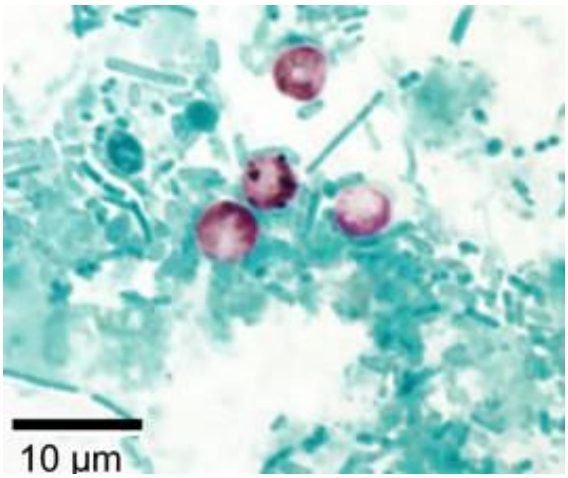


Fig 2.13 *Cryptosporidium spp*. oocysts stained with modified acid-fast at 1000x magnification

Immunological methods can be categorized into either antigen detection or antibody detection. Antigen-detection assays, such as immunofluorescent assays, enzyme-linked immunosorbent assay (ELISA), and immunochromatographic tests, exhibit significantly greater sensitivity compared to microscopy. These approaches have demonstrated good sensitivity and specificity, typically ranging from 93% to 100% in various studies. Antigen detection tests are valuable for diagnosing acute infections, while antibody detection tests are particularly useful in seroepidemiological surveys. Both direct and indirect fluorescent antibody techniques are utilized for antigen-based detection. In recent years, commercial copro-antigen tests, including enzyme immunoassay (EIA) and immunochromatographic dipstick tests (ICT), have been employed for rapid diagnosis. *Cryptosporidium* oocysts can be detected using monoclonal antibodies (mAbs) targeting the oocyst wall antigen (C-mAbs). These mAbs specifically recognize epitopes on the oocyst surface, with many commercially available mAbs raised against *C. parvum*. Commercially available kits for antigen detection utilize antibodies labeled with enzymes, suitable for enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), or immunochromatographic (IC) formats. Coproantigen detection ELISA has reported variable detection limits ranging from 3 × 10^5 to 10^6, comparable to microscopy. However, the sensitivity may vary depending on the specific antibody used in the kit. Despite this, it offers the advantage of high specificity, ranging from 98% to 100%, and the ability to process a large number of samples quickly. An indirect diagnostic method involves demonstrating the presence of antibodies to *Cryptosporidium*-specific antigens in serum, saliva, or fecal samples. This method indicates evidence of infection or exposure. Detection of specific circulating antibodies is particularly beneficial during seroconversion, showcasing titer elevation or antibody isotype switching. Serum antibodies are generated against the 27-kDa and 15/17-kDa antigens on the sporozoite surface post-infection.

Various formats exist for antibody detection, utilizing native or recombinant *Cryptosporidium* antigens. Initially, ELISA employed crude *C. parvum* extracts with lower specificity than Electro Immuno Transfer Blot. However, the specificity has increased with the availability of recombinant *Cryptosporidium* antigens. Studies using various recombinant proteins have shown promising results, with a reported comparable performance of a recombinant 23kDa antigen (Cp23) to ELISA using native 27kDa antigen (Khurana and Chaudhary, 2018).

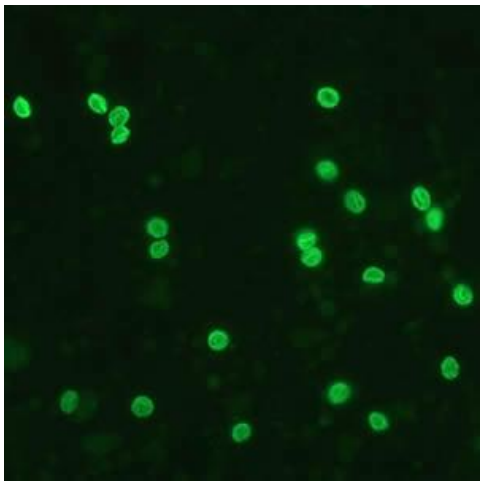


Fig 2.14: *Cryptosporidium* sp. oocysts labeled with immunofluorescent antibodies

The field of diagnostic laboratories has undergone a transformative shift with the introduction of nucleic acid based molecular methods, viz., Polymerase Chain Reaction (PCR). PCR methods offer heightened sensitivity, detecting *Cryptosporidium* in the range of 1 to 10^6 oocysts. These techniques are relatively rapid and carry a significant advantage for speciation, a crucial aspect in epidemiology for understanding potential transmission routes. Several nucleic acid detection methods, such as PCR-restriction fragment length polymorphism (PCR-RFLP), multiplex allele-specific PCR (MAS-PCR), and quantitative real-time PCR, are employed in this context (Khurana

and Chaudhary, 2018). Different gene targets are utilized for species identification, including 18S rRNA, TRAP C1, COWP, Hsp 70, and DHFR genes. Subtype determination is further possible through tools like the glycoprotein GP60 gene, minisatellite and microsatellite markers, and the analysis of extrachromosomal double-stranded RNA elements (Smith, 2007).

The conventional approach for detecting *Cryptosporidium* in food and water samples involves the large volume sampling of 10–1000 liters. This is followed by concentration through methods like filtration, the use of magnetic beads coated with chitin or specific antibodies, etc. Detection typically occurs through indirect fluorescent microscopy or molecular techniques such as PCR (Xiao et al., 2006).

Molecular Characteristics

The 18S ribosomal (SSU RNA) locus has been widely employed for the identification of *Cryptosporidium* spp. and genotypes through techniques such as RFLP and Sanger sequencing (Xiao and Feng, 2017). Due to its multi-copy nature and the presence of both semi-conserved and hypervariable regions, this locus is highly sensitive, efficiently detecting all species and genotypes. Complementary analysis at the Actin and Heat Shock Protein 70 (*hsp70*) loci provides additional support (Sulaiman et al., 2000, Sulaiman et al., 2002). While the 18S locus serves well for detection, more variable loci are essential for tracking transmission. In this regard, the highly polymorphic glycoprotein 60 (*gp60*) gene is crucial (Priest et al., 2000; Strong et al., 2000). A subtyping system has been established based on tandem repeats of the serine-coding trinucleotide TCA, TCG, or TCT at the 5' end of the gene, coupled with significant sequence divergence in non-repeat regions (Xiao and Feng, 2017; Strong et al., 2000). For effective subtyping and transmission tracking, subtype family designations have been assigned for *C. hominis* (Ia, Ib, Id, Ie, If, etc.), *C. parvum* (IIa, IIb, IIc, IId, etc.), and *C. meleagridis* (IIIa, IIIb, IIIc, IIId, etc.). Additional variation

is introduced based on the number of repeats. For instance, the subtype name IIcA5G3 signifies membership in the *C. parvum* subtype family IIc, with five copies of the TCA repeat and three copies of the TCG repeat. Within the IIc subtype family, further distinctions are made based on sequences in the 3' region, denoted as IIcA5G3a and IIcA5G3b (Xiao and Feng, 2017). Standardized methodologies have now been developed for typing *Cryptosporidium* at both the 18S rRNA and *gp60* loci.

Treatment

Despite efforts spanning over three decades to develop specific treatment strategies for cryptosporidiosis, options remain extremely limited. Clearing the host of *Cryptosporidium*, especially in immunocompromised individuals, continues to pose a significant challenge. In immunologically competent individuals, cryptosporidiosis tends to be a self-limiting illness. Supportive treatment involves fluid replacement, and in chronic cases, parenteral nutrition. However, there is a pressing need for more effective therapies, particularly for immunocompromised individuals experiencing severe morbidity. Symptomatic therapy involves addressing fluid replacement, nutrition, and the treatment of diarrhea (Ayuo, 2009). The uptake of Na+ is significantly increased with luminal glutamine compared to glucose, indicating that oral rehydration fluids incorporating glutamine (rather than glucose) could be beneficial in preventing dehydration caused by cryptosporidiosis (Crawford and Vermund, 1988). Anti-diarrheal agents, such as loperamide, diphenoxylate-atropine, somatostatin analogs (octreotide), or opiates, are utilized in conjunction with antiparasitic agents. Paying attention to the nutritional aspects of patient care is crucial as malnutrition can be fatal (Ayuo, 2009). The introduction of highly active antiretroviral therapy (HAART) has significantly reduced the impact of cryptosporidiosis among individuals with HIV. This improvement is attributed to an increase in CD4 cell count, the

restoration of a degree of immunity, and a direct effect of protease inhibitors on host cell invasion and parasite development in vitro, particularly when enhanced with paromomycin. Currently, nitazoxanide (NTZ), a synthetic antiparasitic agent that rapidly hydrolyzes to its active metabolite tizoxanide, is the only FDA-approved drug in the United States for treating cryptosporidiosis in immunocompetent patients, although it is not licensed in Europe. However, there is limited evidence supporting the efficacy of NTZ in immunocompromised individuals and malnourished children (Caccio and Chalmers, 2016).

Prevention and Control Measures

Effective vaccines to control *Cryptosporidium* are currently lacking. Oocysts have been observed to maintain their survival and infectious properties for 8 weeks in a 2.5% aqueous potassium dichromate solution when stored at temperatures between 20 to 25°C. They can persist for 2 to 6 months at 4°C, regardless of the storage method, and for 8 to 9 months when stored in containers with limited air exposure. *Cryptosporidium* oocysts exhibit resistance to various substances, including cresylic acid, sodium hydroxide, iodophore, sodium hypochlorite (2 to 5%), benzalkonium chloride, and aldehyde-based disinfectants. The infectivity of the organism is neutralized by 10% formaldehyde, 5 to 10% household-strength ammonia, and undiluted 70% commercial bleach. Freeze-drying or exposure to temperatures below freezing or above 65°C for 30 minutes can eliminate infectivity. Another study suggests that maintaining the inoculum at 45°C for 10 to 20 minutes can destroy infectivity, implying that pasteurization of milk could effectively prevent *Cryptosporidium* transmission. For individuals treating patients with *Cryptosporidium*, strict hand-washing and enteric precautions (wearing a gown and gloves) are strongly recommended. Contaminated instruments should undergo autoclaving, and surfaces should be treated with commercial bleach for 10 to 15 minutes. Laboratory workers are advised to wear

gloves and employ closed techniques and/or a fume hood when handling potentially infectious material. Travelers are encouraged to avoid consuming untreated water and eating raw or unpeeled foods, particularly salads (Crawford and Vermund, 1988).

Cyclospora cayetanensis

Introduction

Cyclospora cayetanensis is another coccidian parasite widely known as a causative agent of gastrointestinal illnesses in humans. Similar to *Cryptosporidium*, it is mainly transmitted through the fecal-oral route. Among the *Cyclospora* genus, *C. cayetanensis* is the sole species known to infect humans. (Almeria et al., 2019). Usually, *C. cayetanensis* infection manifests with persistent diarrhea, along with symptoms such as loss of appetite, general discomfort, nausea, and abdominal cramps. The initial documented instances of human *Cyclospora* infection were identified in the late 1970s by Ashford, a British parasitologist, in Papua New Guinea (Herwaldt, 2000). *C. cayetanensis* infections have been reported in over 50 countries, with documented outbreaks of cyclosporiasis occurring in 13 of them. (Li et al., 2019). Other species within this genus have been identified in non-human primates, and they are likely to be specific to their respective hosts. The most recently identified species, *Cyclospora macacae*, was documented in rhesus monkeys (*Macaca mulatta*). Additionally, *Cyclospora* oocysts with sequences closely resembling to *C. cayetanensis* have been observed in chimpanzees (*Pan troglodytes*) and cynomolgus monkeys (*Macaca fascicularis*) based on ITS-2 locus analysis (Almeria et al., 2019).

Taxonomy

The genus *Cyclospora*, established by Schneider in 1881, was initially described by Eimer in 1870. In 1994, Ortega et al. officially designated the human pathogenic species as *C. cayetanensis*. *Cyclospora* spp. belong to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eimeriorina, family Eimeriidae, and the genus *Cyclospora*. (Li et al., 2019). Phylogenetic

studies have revealed that the *Cyclospora* genus are closely related to members of the *Eimeria* genus.

Kingdom: Protista

Sub-Kingdom: Protozoa

Phylum: Apicomplexa

Class: Sporozoasida

Sub-Class: Coccidiasina

Order: Eimeriorina

Family: Eimeriidae

Genus: *Cyclospora*

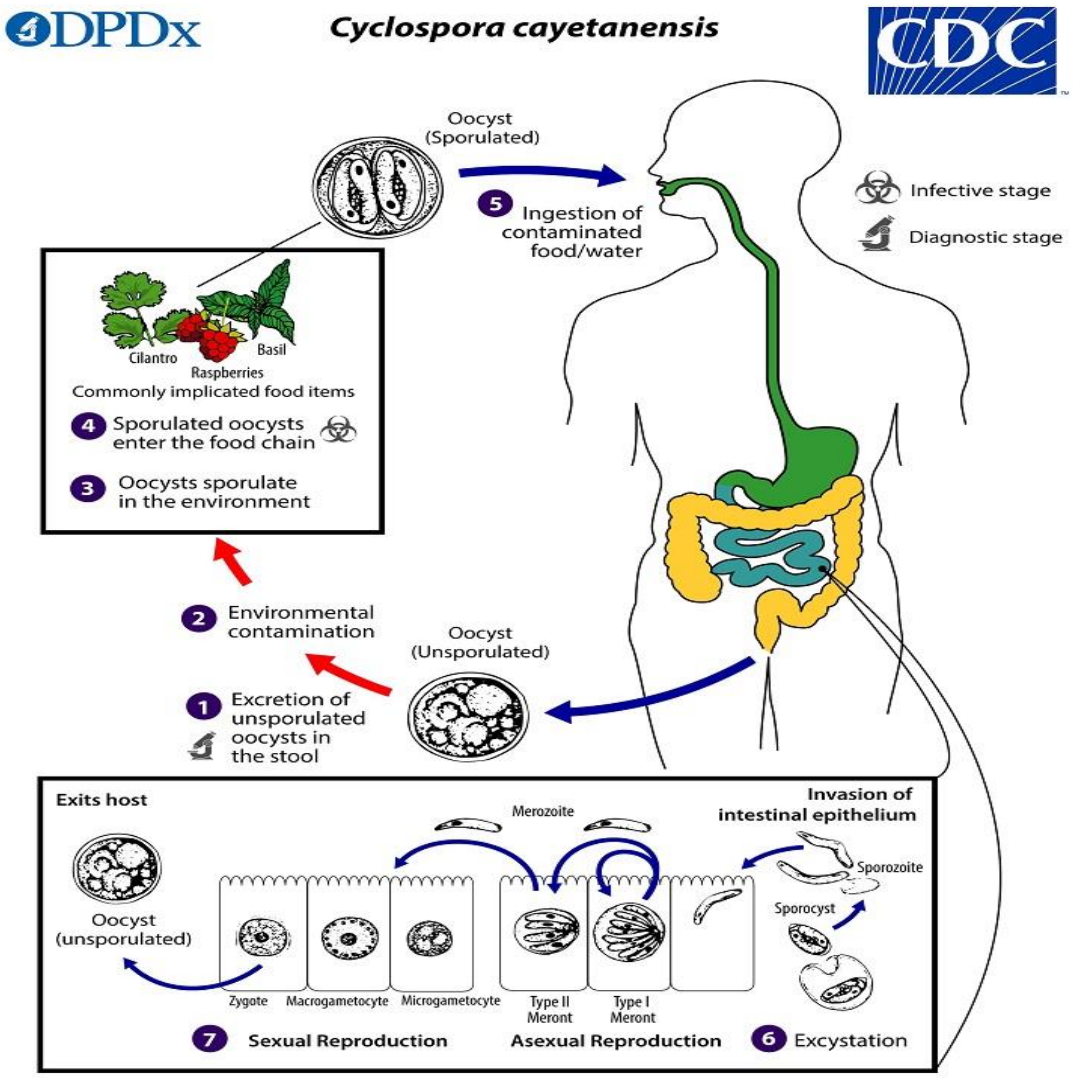
Species: *cayetanensis*

Life Cycle

C. cayetanensis infections primarily spread through the fecal-oral transmission route. Unsporulated oocysts, which are spherical and measure 8-10 μm in diameter, are excreted in feces (Brown and Rotschafer, 1999). In the external environment, freshly excreted oocysts are non-infectious until they have completed sporulation, a process that typically takes a few days to a maximum of a few weeks. This transformation occurs at temperatures ranging from 22 to 30 °C. If oocysts are stored at either 4 °C or 37 °C, sporulation is delayed (Smith *et al*., 1997). The sporulation takes place regardless of whether they are stored in deionized water or a potassium dichromate solution. During sporulation, the sporont divides into two sporocysts, with each

sporocyst containing two elongated sporozoites. (Smith *et al*., 1997). During this period, transmission can occur through contaminated food or water. When sporulated oocysts present in food, water, or soil are consumed by a new host, the mature oocysts typically undergo excystation in the small intestine, releasing sporozoites to invade the epithelial cells of the upper part of small intestine. (Ortega and Sanchez, 2010).

Occurrence of both asexual and sexual stages within a single host indicates that the life cycle of this microorganism can be entirely accomplished within that host. (Ortega *et al*., 1997). Upon ingestion of sporulated oocysts, sporozoites emerge in the intestinal lumen and invade the enterocytes of the duodenal and jejunal epithelium. Within these cells, sporozoites undergo a transformation into trophozoites, which then give rise to two distinct types of meronts, also known as schizonts. (Almeria et al., 2019). The intracellular development begins with the formation of the intracytoplasmic parasitophorous vacuoles within the cells of the intestinal epithelium. (Sun *et al*., 1996; Ortega and Sanchez, 2010), sometimes found in biliary epithelium (Zar *et al*., 2001). Asexual reproduction leads to the generation of two types of meronts: Type I meronts produce 8–12 merozoites, which are 3–4 μm long and subsequently infect nearby epithelial cells. This type of asexual replication is typically highly productive. Type II meronts develop later and release 4 merozoites, which measure 12–15 μm in length, to invade neighboring cells. Some of these meronts give rise to macrogametes, while others undergo multiple fission events, resulting in the formation of microgametocytes having flagellated microgametes. (Ortega *et al*., 1997).



When freshly passed in stools, the oocyst is not infective 1 (thus, direct fecal-oral transmission cannot occur; this differentiates *Cyclospora* from another important coccidian parasite, *Cryptosporidium*). In the environment 2 , sporulation occurs after days or weeks at temperatures between 22°C to 32°C, resulting in division of the sporont into two sporocysts, each containing two elongate sporozoites 3 . The sporulated oocysts can contaminate fresh produce and water 4 which are then ingested 5 . The oocysts excyst in the gastrointestinal tract, freeing the sporozoites, which invade the epithelial cells of the small intestine 6 . Inside the cells they undergo asexual multiplication into type I and type II meronts. Merozoites from type I meronts likely remain in the asexual cycle, while merozoites from type II meronts undergo sexual development into macrogametocytes and microgametocytes upon invasion of another host cell. Fertilization occurs, and the zygote develops to an oocyst which is released from the host cell and shed in the stool 7 . Several aspects of intracellular replication and development are still unknown, and the potential mechanisms of contamination of food and water are still under investigation.

Fig 2.15: Complete life cycle of *Cyclospora cayetanensis* and transmission route

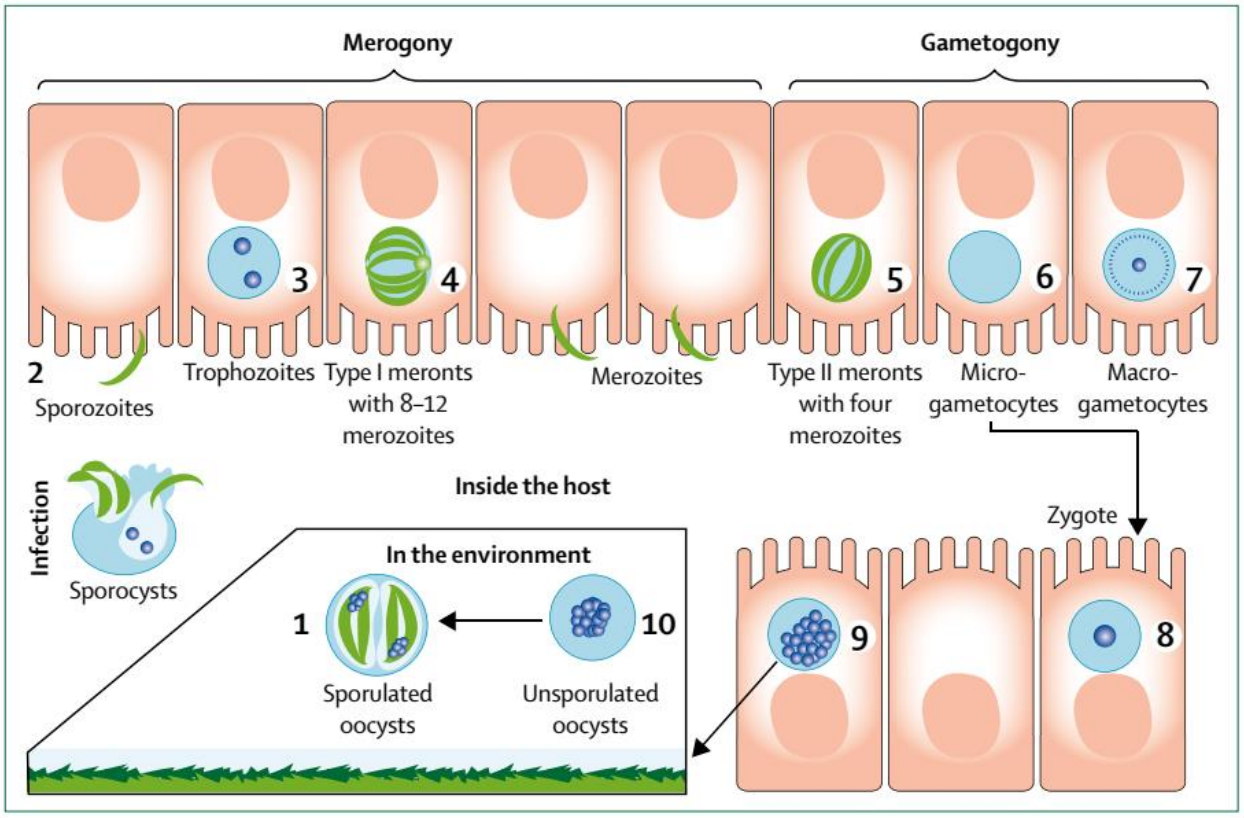


Fig 2.16: Asexual and sexual stages of *Cyclospora cayetanensis* inside host gut

In the sexual stages, the macrogametocyte is fertilized by the microgametocyte, resulting in the formation of a zygote. Upon fertilization, a resilient wall is created, leading to the development of oocysts within enterocytes. These oocysts are then expelled from the host into the environment via feces as unsporulated oocysts (Shields and Olson, 2003; Ortega and Sanchez, 2010). The prepatent period is typically estimated to be around one week. (Li et al., 2019).

Morphology

C. cayetanensis oocysts, when observed under light microscopy, exhibit a spherical shape with a diameter ranging from 8 to 10 µm. The internal structure of these oocysts appears homogenous and lacks distinct protoplasmic features. Upon sporulation, each oocyst contains two ovoid

sporocysts, and each of these sporocysts houses two sporozoites. Notably, the staining characteristics of *Cyclospora* oocysts using the Ziehl-Neelsen acid-fast stain can vary. The oocysts can exhibit different staining patterns when subjected to Ziehl-Neelsen acid-fast stain. Some oocysts take on a dark red hue with a mottled appearance, while others appear pink, and some remains unstained, presenting as non-refractile, glassy spheres against a blue-green background. Furthermore, the inherent autofluorescence of these oocysts facilitates their clear visibility in clinical samples when examined using epifluorescence microscopy under a 330-380 nm ultraviolet (UV) filter (CDC, 2019).

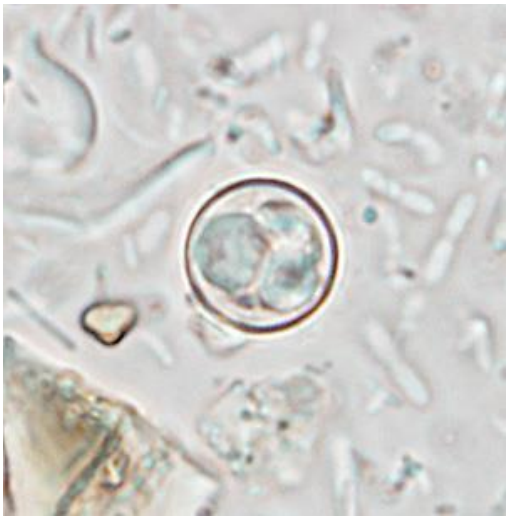


Fig 2.17: *C. cayetanensis* oocysts at 1000x magnification, unstained wet mount

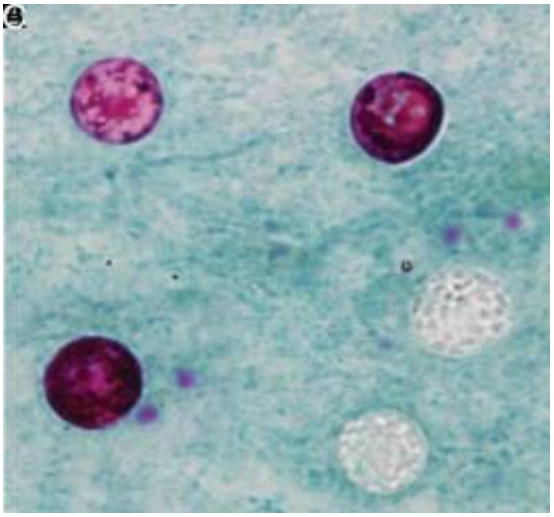


Fig 2.18: *C. cayetanensis* oocysts at 1000x magnification, stained with modified acid-fast stain; variability of staining of oocysts observed

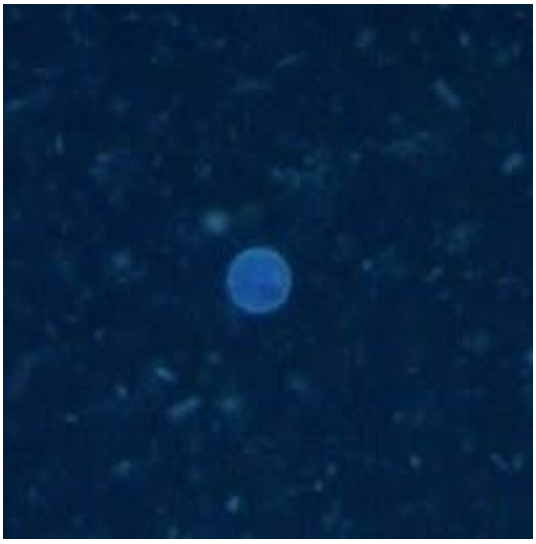


Fig 2.19: *C. cayetanensis* oocysts under UV microscope, 330-380 nm UV filter

Transmission

C. cayetanensis primarily spreads through the consumption of contaminated food, such as fresh produce like berries and leafy greens, being the main culprits due to their difficulty in being thoroughly cleaned and often eaten raw. Foods that are pasteurized or thoroughly cooked before consumption do not typically lead to infections. Recent studies have revealed contamination in ready-to-eat and pre-packaged/bulked vegetable products in Canada and Europe, suggesting that current sanitation processes do not always ensure food safety, particularly with fecal-origin parasites (Dixon et al., 2013; Caradonna et al., 2017). The global food trade, which includes the import of fresh produce from developing regions, plays a significant role in transmitting *C. cayetanensis*, especially in developed countries like the USA. Raspberries, blackberries, mesclun, bagged mixed greens, snow and snap peas, cilantro, and basil have been implicated in numerous *Cyclospora* outbreaks worldwide. Historically, cilantro and raspberries have been key components in cyclosporiasis outbreaks in North America. Additionally, herbs like Basil, salad greens like lettuce and romaine-carrot mixes have been linked to outbreaks in the USA and Europe (Almeria

et al., 2019). *C. cayetanensis* can also be transmitted through water contaminated with fecal matter, particularly in endemic areas where drinking water sources have been identified as a risk factor (Bhandari et al., 2015). The oocysts have been detected in various types of water, including chlorinated water and wastewater, suggesting potential transmission through drinking and recreational water. Waterborne transmission can be a significant concern, especially in areas with inadequate water and sewage treatment systems. Oocysts can persist through treatment processes and are unaffected by chlorine and other disinfectants (Rabold et al., 1994).

Soil is another potential source of infection and transmission for *C. cayetanensis*. Inadequate sanitation practices leading to soil contamination are a significant determinant for infection (Chacín-Bonilla, 2008). Contact with contaminated soil has been identified as a risk factor for *C. cayetanensis* infections in both developing and developed countries. Soil contamination has been observed in various areas, emphasizing the importance of improved sanitary practices to prevent infections.

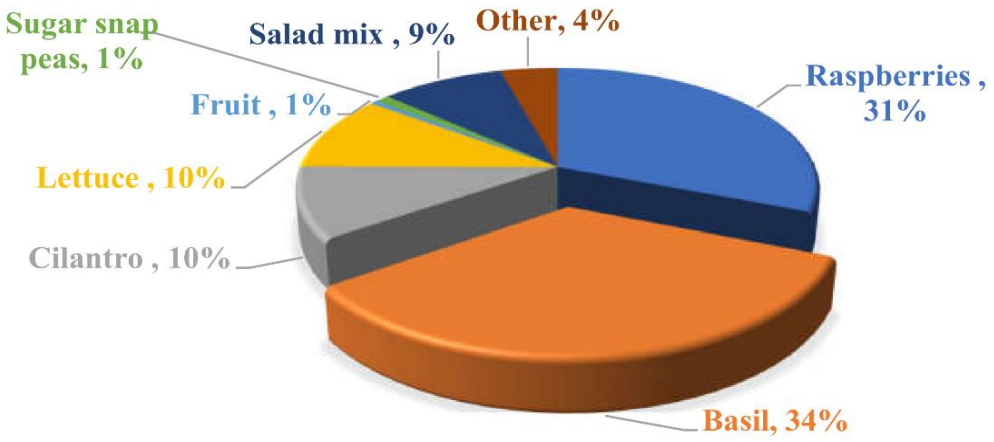


Fig 2.20: Fresh produce as a vehicle for cyclosporiasis transmission

Clinical Features

In humans, cyclosporiasis typically presents with clinical symptoms that include recurrent, profuse watery diarrhea, along with feelings of malaise, nausea, anorexia, cramping, and occasional periods of apparent symptom relief. Healthy individuals who have ingested sporulated oocysts commonly experience mild to moderate, self-limiting diarrhea. However, individuals with compromised immune systems may endure severe intestinal damage and prolonged episodes of diarrhea (Mansfield and Gajadhar, 2004). In certain cases, there may also be a low-grade fever and malabsorption of d-xylose (Shields and Olson, 2003). Asymptomatic infections are prevalent in areas where the disease is endemic. *C. cayetanensis* infections result in noticeable histological alterations in the intestine, such as acute or chronic inflammation, disruption of the surface epithelium, villous atrophy, crypt hyperplasia (Connor et al., 1993), and a significant influx of lymphocytes within the lamina propria and epithelial cells (Ortega et al., 1997; Wiwanitkit, 2006). In addition to gastrointestinal symptoms, *C. cayetanensis* can also infect the biliary tract (Sifuentes-Osornio et al., 1995), potentially leading to acalculous cholecystitis in individuals with acquired immunodeficiency syndrome (AIDS) (Zar et al., 2001). Furthermore, *C. cayetanensis* infection has been linked to various secondary conditions, including reactive arthritis syndrome, Reiter syndrome, and Guillain-Barre syndrome (Li et al., 2019).

Epidemiology

C. cayetanensis infections have been documented in humans across more than 56 countries worldwide, spanning all five inhabited continents. The initial recorded outbreak of *C. cayetanensis* infection, referred to as an 'alga-like organism' at the time, occurred among 55 British expatriates experiencing prolonged diarrhea in Nepal in 1989 (Shlim et al. 1991). The first reported outbreak

of diarrheal illness associated with *Cyclospora* infection in the United States took place in 1990 (Huang et al 1995). To date, North America has reported more than 11,500 cases of these infections. A total of 13,845 cases of *C. cayetanensis* infection have been documented in humans, encompassing cases found during epidemiological studies (5,478), outbreak investigations (6,557), and case reports (1,810). The global prevalence of *C. cayetanensis* in humans stands at 3.55%. Notably, Asia (5.63%), and Africa (5.33%) exhibit a higher prevalence compared to the America (3.03%) and Europe (1.28%). Notable examples include Nepal (13.68%) and India (5.58%) in Asia, Madagascar (16.59%) and Egypt (5.74%) in Africa, and Venezuela (9.90%), Peru (6.87%), and Haiti (8.47%) in the America (Li et al., 2019). Numerous outbreaks have been documented among expatriates to developing countries. Among the notable recent outbreaks, one significant occurrence involved visitors returning from the Riviera Maya region of Mexico in 2015. This outbreak resulted in 79 cases in the United Kingdom (UK) and 97 cases in Canada (Nichols et al., 2015). Multiple outbreaks of *C. cayetanensis* occurred in diverse settings, including a group of Dutch individuals visiting Bogor, Indonesia, where 50% of the participants (14 out of 29) later tested positive. Additionally, Spaniard workers traveling for international aid-related purposes in Guatemala experienced an outbreak, while a separate small outbreak emerged among 3 Polish businessmen during their trip to Indonesia (Puente et al., 2006; Bednarska et al., 2015).

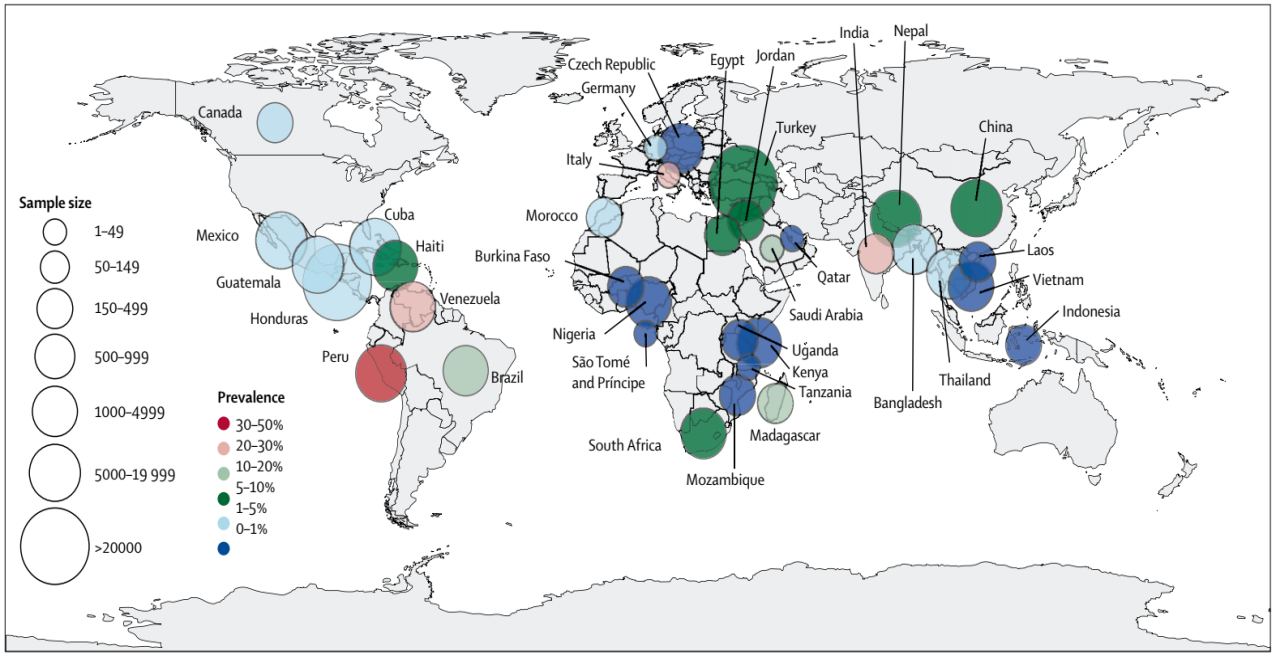


Fig 2.21: Estimated prevalence and distribution of *Cyclospora* infection in immunocompetent people, globally

Elevated prevalence rates were noted among immunocompromised individuals, primarily consisting of HIV/AIDS patients experiencing diarrhea. In a study involving 3,340 such individuals, prevalence levels ranged from 0% to 36%, with an average of 4.5%. The prevalence levels ranged from a low of 0.5% among 2,540 patients in Thailand to a high of 22.2% among 256 patients in India. The highest prevalence was documented in Peru, reaching 41.6%. Another systematic review, encompassing 27 studies from 14 sub-Saharan countries, unveiled an overall prevalence of 18% (Almeria et al., 2019)

Seasonality

C. cayetanensis infection exhibits a distinct and often seasonal pattern worldwide, influenced by various factors, including human activities, environmental contamination, and specific sporulation

conditions in different regions. This seasonality is not solely tied to rainfall, as it varies markedly even in dry environments. The incidence of *C. cayetanensis* infection tends to rise during warm periods with maximum rainfall in countries like Guatemala, Honduras, Mexico, Jordan, Nepal, and China (Bern et al., 2002; Orozco-Mosqueda et al., 2014; Bhandari et al., 2015). However, in Peru and Turkey, the infection is more prevalent during drier and hotter months (Madico et al., 1997; Turgay et al., 2007). In Haiti, infections occur during the driest and coolest times of the year, or during the cooler wet season in Indonesia (Eberhard et al., 1999; Fryauff et al., 1999). In India, clinical cases are more frequent in the summer before the rainy period (Tuli et al., 2008). These variations in seasonality are complex and cannot be attributed to a single common factor. It's important to note that the cooler months in Haiti have temperatures similar to the warmest months in Guatemala City and Kathmandu. Additionally, in Vietnam, produce contamination increases before the rainy season (Tram et al., 2008), while in Guatemala, clinical cases surge during the raspberry harvest from April through June (Bern et al., 2002). Notably, the seasonal pattern observed in endemic areas like Mexico aligns with the peak clinical prevalence observed in the USA (Hall et al., 2011).

Detection Method

C. cayetanensis infection can be diagnosed through various laboratory methods. One simple approach involves direct examination of wet-mount preparations of fecal samples under light microscopy. An alternative automated method for detecting *C. cayetanensis* in human fecal specimens has been developed based on oocyst morphology and autofluorescence characteristics utilizing UV epifluorescence microscopy (Dixon et al., 2005). For the detection of *Cyclospora* oocysts, the use of Modified Ziehl-Neelsen acid-fast staining is recommended. Other staining methods like (modified) Kinyoun acid-fast staining, trichrome staining, carbol fuchsin staining,

(modified) safranin staining, and lactophenol cotton blue staining have been used in the past to identify *Cyclospora* oocysts in fecal smears. However, it's important to note that these morphology-based detection methods require a higher parasite burden, which can result in both false positive and false negative results (Li et al., 2019).

Modern molecular methods enable precise and sensitive diagnosis in clinical and epidemiological settings, using samples from feces, tissues, food, and the environment. Accurate detection of *Cyclospora*, identification of *Cyclospora* species, and diagnosis of cyclosporiasis are vital for clinical management, epidemiological understanding, prevention, and control of the disease. Nucleic acid-based techniques have significantly improved diagnostic and analytical performance. Polymerase chain reaction (PCR) plays a pivotal role by amplifying specific genetic loci from minute amounts of *Cyclospora* genomic DNA. PCR commonly targets genetic loci within the small subunit of the nuclear ribosomal RNA (18SrRNA) gene, the second internal transcribed spacer (ITS), or the 70 kDa heat shock protein (Hsp70) gene. Various PCR-based methods, including microsatellite analysis, restriction fragment length polymorphism analysis, mutation scanning, and direct DNA sequencing, have also been developed for detecting, identifying, and characterizing *Cyclospora* (Giangaspero and Gasser, 2019). Recent progress in diagnosing infections involves the application of a multiplex PCR method carried out on a Luminex platform. This method focuses on the small subunit ribosomal RNA (rRNA) gene of *Cyclospora*. It has exhibited impressive sensitivity and specificity, ranging from 87% to 100%. Additionally, a recent two-step nested PCR method has been developed, targeting the 70-kDa heat shock protein of *Cyclospora* (Legua and Seas, 2013). Four real-time PCR assays were also evaluated for *C. cayetanensis*, with two using a species-specific TaqMan probe to detect unique regions in the small subunit ribosomal RNA (18S rRNA) gene and the other two relying on DNA-binding dyes and

melting curve analysis of amplicons. All of these assays achieve specific identification in a single PCR. Comparing these four assays for detecting *C. cayetanensis* in human stool samples that tested positive by microscopy showed high diagnostic specificity (98-100%) and sensitivity (93-100%). The TaqMan probe assay displayed the best specificity (100%) and sensitivity (100%) and appears well-suited for the molecular detection of *C. cayetanensis* in stool samples (Giangaspero and Gasser, 2019).

Molecular characteristics

The whole genome of *C. cayetanensis* is estimated to have a total length of approximately 44Mbp with a GC content of around 52%, and it contains approximately 7,500 genes (Liu et al., 2016). A comparative genomic analysis has shown that *C. cayetanensis* shares metabolic and invasion components resembling those of coccidia but possesses unique surface antigens. Analysis of small subunit ribosomal RNA (SSU rRNA) gene sequences has revealed minimal genetic diversity among *C. cayetanensis* isolates worldwide. However, the internal transcribed spacer (ITS) sequences in *C. cayetanensis* exhibit high variability within and between samples, with this variability not being associated with geographic origins (Olivier et al., 2001). This ITS sequence variability occurs at the individual-genome level, and regions of the 70kDa heat shock protein (HSP70) locus do not display genetic polymorphism, as observed in a previous study. Researchers have explored the polymorphic regions of the *Cyclospora* genome to gain insights into the microorganism's mode of infection and epidemiology (Sulaiman et al., 2013). A multilocus sequence typing tool for *C. cayetanensis* has been developed based on its whole genome, involving five microsatellite loci (Guo et al., 2016). Notably, geographic clustering has been observed among human *C. cayetanensis* isolates worldwide exploiting these loci (Li et al., 2017). Two novel similarity-based classification algorithms for *C. cayetanensis* have been created, incorporating

Bayesian and heuristic components to infer the relatedness of pathogen isolates (Barratt et al., 2019).

The apicoplast genome of *C. cayetanensis* is approximately 34,000 base pairs in size and encodes approximately 65 genes, with a GC content of around 22% (Tang et al., 2015; Liu et al., 2016). The apicoplast genome is circular and contains the complete machinery for protein biosynthesis, including two inverted repeats with slight differences in the LSU rRNA gene sequences (Tang et al., 2015). A comparative genomic analysis has revealed a high-nucleotide sequence similarity of 85.6% between *C. cayetanensis* and *Eimeria tenella*, and a phylogenetic analysis of apicoplast genomic sequences has confirmed the genetic similarities between avian *Eimeria* spp. and *C. cayetanensis* (Li et al., 2019).

Prevention and Control

Infections caused by *C. cayetanensis* can be prevented through improved personal hygiene and better sanitary conditions, with the goal of eliminating potential fecal-oral transmission from contaminated food, water, and the environment in endemic areas. Another preventive measure is to avoid consuming raw, fresh produce, especially in areas where the infection is prevalent.

Methods like gamma-irradiation (at 1.0 KGy and higher) (Lee and Lee, 2001) effectively decontaminated raspberries, and hydrostatic pressure (HPP) (550 MPa at 40°C for 2 minutes) (Kniel et al., 2007) demonstrated efficacy in raspberries and basil. Sodium dichloroisocyanurate (NaDCC) has been used in disinfection studies against various intestinal protozoa, including *C. cayetanensis*. It involved dipping raw vegetables and fruits into a NaDCC solution (1g/liter), resulting in reduced parasite numbers (El Zawawy et al., 2010).

For water used in drinking, food preparation, and washing of raw produce, it is advisable to either boil or filter it. As mentioned earlier, *C. cayetanensis*, and coccidia in general, exhibit high resistance to sanitizers and disinfectants. Thus, controlling potential sources of contamination in fields, packing houses, and among farm workers is crucial for preventing *C. cayetanensis* infections, especially in endemic areas. Access to proper toilet facilities, thorough hand washing, and the appropriate disposal and treatment of human sewage are essential practices. Workers displaying symptoms of gastroenteritis should be prevented from having contact with vegetables or food. It is important to note that there is currently no vaccine available to protect humans against this particular coccidian infection (Almeria et al., 2019).

Treatment

Treatment with trimethoprim–sulfamethoxazole (TMP–SMX), at a dosage of 160 mg trimethoprim and 800 mg sulfamethoxazole, administered twice daily for a duration of 7 to 10 days, has demonstrated efficacy in the eradication of *Cyclospora* infection. This treatment approach has proven successful in various studies, including those involving HIV patients and individuals with AIDS-related biliary disease. TMP–SMX, also known as co-trimoxazole, is a highly effective option, and many studies have reported a low recurrence rate (Li et al., 2019). It's worth noting that norfloxacin, metronidazole, tinidazole, and quinacrine have shown ineffectiveness in several human cyclosporiasis studies (Escobedo et al., 2009). In a case where a patient with a sulfonamide allergy, did not respond to ciprofloxacin, a novel thazolide treatment, nitazoxanide, was administered, resulting in symptom improvement after seven days. Nitazoxanide was also employed to address mixed infections involving intestinal protozoa and helminths, including *Cryptosporidium* spp. and *C. cayetanensis*. The efficacy of nitazoxanide for

cyclosporiasis ranged from 71% to 87%. Importantly, nitazoxanide exhibited excellent tolerability, with no reports of serious adverse effects (Almeria et al., 2019).

Chapter 3

DETECTION OF THE ENTERIC PROTOZOAN PARASITES FROM DIARRHEAL STOOL SAMPLES OBTAINED IN HOSPITAL BASED SURVEILLANCE

3.1. Introduction

Diarrhea stands as the second most prevalent cause of illness and mortality in developing nations, ranking as the second leading cause of death from infectious diseases among children aged five years and below, contributing to 19% of global child fatalities and 63% of the total global diarrhea burden (Mukherjee et al., 2009; Zhang et al., 2016). The World Health Organization (WHO) projects an annual occurrence of 1.7billion diarrhea cases, predominantly concentrated in tropical regions (WHO, 2017). Diarrheal disease constitutes 3.6% of the overall global burden of disability-adjusted life years (Huang et al., 2018). According to WHO definition of diarrhea states as the excretion of three or more loose or liquid stools/ day, than typical for the individual (WHO, 2017). Diarrhea is fundamentally a disrupted movement of ions and water, guided by an osmotic gradient. In normal circumstances, the gastrointestinal tract exhibits a significant ability to absorb fluid and electrolytes. Enteric pathogens have the capability to disrupt this equilibrium, causing a shift towards net secretion and resulting in diarrheal disease (Hodges & Gill, 2010). Therefore, it is commonly presented as a symptom of an infection within the intestinal tract, and this infection can be attributed to a range of bacterial, viral, and parasitic agents. The spread of the infection occurs via the intake of contaminated food or water, or through person-to-person transmission due to inadequate hygiene (WHO, 2017).

Human infections with intestinal parasites have been documented since prehistoric times, and their coexistence with humans has persisted throughout history. The nature of this association has, however, been influenced by global shifts in the socio-cultural landscape of human societies. In contemporary times, parasitic diseases are commonly linked to underdeveloped countries, with the developed world traditionally considering itself "an island free of parasites/worms". Nevertheless, the emergence of immunocompromised populations, coupled with an increase in life expectancy,

has led to a resurgence of parasitic infections in the developed world. The belief that human parasitic diseases are exclusive to underdeveloped regions is therefore, a misconception. Various intestinal parasites are widespread in different regions across the globe. Major contributors to the global burden of intestinal parasitic diseases include *Entamoeba, Giardia, Cyclospora, Cryptosporidium, Ascaris, Trichuris* and *Toxoplasma* (Alum et al., 2010). The occurrence of most enteric infections is influenced by environmental factors, exhibiting restricted geographical distribution and seasonal patterns that are closely tied to the levels of sanitation and hygiene, along with access to clean drinking water. The standards of sanitation, hygiene, and access to safe drinking water are intricately linked to economic development. Consequently, these factors have frequently correlated with the prevalence of numerous parasitic pathogens in developing countries, marking them as endemic regions over time (Petri et al., 2008). Multiple reports have indicated that infections caused by intestinal parasites pose a public health concern in the Indian population. The Gangetic plain of West Bengal, characterized by ample rainfall, and moist climate and soil texture, create an optimal environment for the survival and transmission of protozoan pathogens (Mukherjee et al. 2013b). Metropolitan areas of the state have undergone rapid socioeconomic development in recent years, attracting a large influx of immigrants, primarily from neighboring countries. During a year-long hospital-based survey conducted in Kolkata in 2008, a higher prevalence rate of commonly occurring protozoan pathogens was revealed, including *Giardia lamblia* (13.3%), *Cryptosporidium* spp. (7.6%), and *Entamoeba histolytica* (4.6%) (Mukherjee et al., 2009). This information dates back almost a decade, and the current status regarding the prevalence of enteric protozoan parasites causing diarrhea in this region remains unknown, as there has been no long-term study conducted on the population experiencing diarrhea. In this chapter,

we aimed to investigate the present scenario concerning the burden of protozoan parasites that impact the population, leading to symptomatic diseases, specifically diarrheal illnesses.

3.2. Materials and Methods

3.2.1. Sample Collection and ethical statement

Approval for this study was granted by the Institutional Human Ethics Committee of the Indian Council of Medical Research-National Institute of Cholera and Enteric Diseases (ICMR-NICED), with the IRB Number A-1/2015-IEC. All participants provided informed consent, signifying their voluntary involvement in the study. In the case of children, consent was obtained from their caregivers, i.e., parents or guardians.

This cross-sectional study was conducted at Infectious Disease and Beliaghata General Hospital (ID & BG Hospital) and Dr. B C Roy Post Graduate Institute of Paediatric Sciences, focusing on patients admitted with diarrheal symptoms from October, 2017 to February, 2020. As prominent government hospitals serving as tertiary referral centers for diarrheal diseases in the southeastern zone, the study encompassed a diverse patient population from various regions of West Bengal. The study followed a systematic strategy for sampling, wherein, on two randomly chosen days each week, every fifth patient presenting with diarrhea or dysentery was included in the study. This method guaranteed an unbiased representation of patients with respect to their gender and age during selection process. A total of 6320 fecal samples were collected from patients upon admission by trained medical professional in a sterile container before the initiation of antibiotic treatment. These sample containers were marked with unique identification numbers. These samples were promptly transported to the laboratory within 30 minutes for processing.

3.2.2. Data Collection

Patient demographic information was gathered from the hospital case record files (CRF), while data regarding coinfection with other bacterial or viral pathogens alongside parasites were retrieved from the institutional database of ICMR-NICED. Observational Meteorological data (rainfall) for the region during the study were obtained from the India Meteorological Department database (https://mausam.imd.gov.in). The rainy season, designated as the monsoon, occurs from June to September, with the highest precipitation (mm). The dry season, referred as winter, includes December-February with the least rainfall. The summer season or pre-monsoon, covered March to May, with the highest mean temperature (°C). Post-monsoon includes the months with the lowest mean temperature: October-November.

3.2.3. Sample Preparation

Prior to microscopic examination, the samples underwent concentration using the formol-ether concentration technique (CDC, 2016). Each sample was divided into two separate aliquots: one in its raw stool form (without preservative) and the other dispersed in RNAlater at a ratio of approximately 1:6 (stool: RNAlater) (Carruthers et al., 2019). Both aliquots were then stored at -80°C. The RNAlater-preserved aliquot functioned as a backup in case the fresh fecal sample did not yield enough DNA, or in the event of delays in isolating DNA after the samples were received.

3.2.4. Formalin Ethyl Acetate Concentration method

Each fecal specimen was agitated with an applicator stick to mix homogenously. Then, 5 ml of the fecal suspension (adjusted based on its consistency) is strained through wetted cheesecloth, placed over a disposable paper funnel into a 15 ml conical centrifuge tube. 10% formalin was added through the debris on the gauze to bring the volume in the centrifuge tube to 15 ml. The preparation

was centrifuged 500 g for 10 minutes. The supernatant is decanted. Subsequently, 10 ml of 10% formalin is added to the sediment, and thorough mixing was carried out with the applicator stick. Next, 4 ml of ethyl acetate was added, and tube was sealed with parafilm. The tube was then shaken vigorously in an inverted position for 30 seconds. Again, centrifugation was carried out at 500 g for 10 minutes. The top layers of supernatant were decanted and several drops of 10% formalin are added to resuspend the concentrated specimen. Subsequent testing procedures to detect enteric parasites was carried out.

3.2.5. Preparation of wet mount slides with Iodine

First, to prepare the iodine solution following reagents were required: 5g of powdered iodine crystals, 10g of potassium iodide (KI) and 100ml of distilled water. The distilled water was added to dissolve the potassium iodide. The iodine crystals were then slowly added to the solution. The resulting liquid was filtered and stored in an amber-coloured bottle.

A drop iodine solution was placed on a spotless glass slide. Then a small volume of concentrated fecal material was mixed with it and a thin smear was prepared onto the slide. The wet smear was covered with a cover slip. A CX41 phase contrast microscope (Olympus, Tokyo, Japan) with a 40× objective lens was used to carefully inspect each slide for the presence of cysts of protozoan parasites.

3.2.6. Trichrome staining method

Stained fecal films are largely accepted as the most effective approach for identifying intestinal protozoa in stool samples. The use of permanent stained smears improves the detection of cysts and trophozoites while also being a permanent record of the protozoa encountered. The Wheatley Trichrome process, utilized for faecal specimens, is a modification of Gomori's original staining

procedure for tissue samples. It has various advantages over wet mount preparations, including the ability to detect microscopic protozoa that may have gone undetected in both unconcentrated and concentrated samples.

The following reagents were required for staining procedure: 1) 70% Ethanol plus iodine: Iodine crystals were combined with 70% alcohol to create a dark stock solution. The stock solution was diluted with 70% alcohol before use, resulting in a solution with a strong tea color, 2) 70% Ethanol, 3) commercially purchased research grade Trichrome Stain (Sigma Aldrich, USA), 4) 90% Acid Ethanol: this solution was prepared by mixing 99.5 ml of 90% ethanol and 0.5 ml of glacial acetic acid, 5) 95% ethanol, 6) 100% ethanol, 7) Xylene or xylene substitute.

A small quantity of concentrated fresh fecal sample was applied to make a thin smear on microscope slides. These slides are then allowed to dry using a slide heater set to 60°C. The dried slide was immersed in a solution of 70% ethanol plus iodine for a duration of 10 minutes. Following that, the slide was transferred to 70% ethanol solution and left for 5 minutes. Subsequently, the slide underwent a 3-minute immersion in a second 70% ethanol solution. Then the slide was immersed in Trichrome staining solution for 10 minutes. After the application of stain, a brief distaining step in 90% ethanol plus acetic acid was carried out lasting 1 to 3 seconds. Next, multiple steps of rinsing were conducted using 100% ethanol with each change lasting 3 minutes. Following this, the slide was put in two changes of xylene for 10 minutes. Finally, the slide was mounted with a coverslip using a mounting medium, such as permount. Microscopic examination of the resulting stained smear was carried out utilizing the 40× and 100× objectives. In every staining run, a control slide is incorporated, having a preserved specimen of an already identified protozoan, such as *Entamoeba* spp., *Giardia lamblia* etc.

3.2.7. Kinyoun Staining Method

Kinyoun staining method is useful for identifying coccidian oocysts (such as *Cryptosporidium, Cyclospora*, and *Cystoisospora*) that are difficult to detect using trichrome staining. This technique, unlike the Ziehl-Neelsen Acid-Fast Stain, does not require the heating step during the staining process and hence, often referred to as the cold method. The required reagents for this process were following: 1) absolute methanol, 2) 10% acid alcohol solution: 10 ml of sulfuric acid + 90 ml of absolute ethanol, 3) Kinyoun's carbol fuchsin (Himedia, India), 4) methylene blue (Himedia, India).

A thin smear of concentrated fecal sample was first prepared and heat fixed. The slide was next fixed with methanol for 30 seconds. After that, the slide was subjected to carbol fuchsin stain for 5 minutes. The slide was then rinsed with distilled water. Subsequently, the slide was destained with 10% acid alcohol solution for 2 minutes. The slide was rinsed with distilled water to remove residual acid alcohol to stop over destaining. The smear was then counterstained with methylene blue for 2 minutes following a water rinse. Finally, a 100x oil immersion objective was used to examine the slide under a microscope. A control slide containing an identified specimen of an acid-fast protozoan species, such as *Cryptosporidium* spp. or *Cyclospora* spp, was included for comparison in each microscopic observation.

3.2.8. Molecular Detection of enteric protozoan parasites

Following the microscopic inspection, the presence of intestinal parasites in stool samples was confirmed by nucleic acids based molecular analysis via Polymerase Chain Reaction (PCR), which targets specific genetic loci. Total DNA was first extracted from stool samples and quality of DNA was checked before setting up PCR reaction.

3.2.8.1. Total DNA isolation from fecal samples

Using QIAmp DNA stool mini kit (Qiagen, USA), total genomic DNA from fecal sample was extracted following the manufacturer's protocol. The eluted DNA was quantified and checked for purity, following storage at -20°C.

3.2.8.2. PCR detection of *Entamoeba* spp.

To confirm the presence of *Entamoeba* spp in clinical sample, a common primer pair specific to the genus, namely EntaF and EntaR, was used (Table 3.1.). This primer set targets SSU rDNA locus for all *Entamoeba* species. These primers were designed from the conserved region of *Entamoeba* species complex i.e., *Entamoeba histolytica/ Entamoeba dispar/ Entamoeba moshkovskii,* that commonly infect humans. The PCR (25 µl) reaction included approximately 100 ng of template DNA, 1X buffer containing 1.5 mM $MgCl_2$ (TaKaRa, Japan), 200 µM of dNTP (TaKaRa, Japan), 0.2 µM of each primer (Eurofins, Luxembourg), 2.5 U of TaKaRa Taq (TaKaRa, Japan), and nuclease-free water. The PCR conditions were: 3 minutes of initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and amplification step at 72°C for 30 seconds. Finally, a 7-minute extension step at 72°C was performed. After that, the amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualized with a UV-transilluminator, following ethidium bromide staining. To verify the results, the set includes both positive (genomic DNA) and negative or no template controls.

3.2.8.3. PCR detection of *Cryptosporidium* spp.

Initial genus-specific conventional PCR screening for commonly affecting *Cryptosporidium* spp. was performed using the Hsp70 gene as the target. The 25 µl PCR reaction mixture consisted of

2.5 U of TaKaRa rTaq polymerase (TaKaRa, Japan), 1X buffer, 2 mM MgCl2, nuclease-free water, 200 µM of dNTPs (TaKaRa, Japan) and 0.2 µM of each of the forward (CrHSP70_F) and reverse (CrHSP70_R) primers (Eurofins, Luxembourg) (Table 3.1). PCR condition involved initial denaturation at 94°C for 3 minutes, then 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 of 5 minutes at 72°C and hold at 4°C are included in the cycle. To validate the results, both positive (genomic DNA) and negative controls (containing all PCR reagents but no DNA template) were included in the set.

3.2.8.4. PCR detection of *Giardia lamblia*

A conventional PCR method (Mukherjee et al. 2009) was used to detect the presence of *G. lamblia* in stool, targeting a 218-bp region of the parasite's beta giardin (*bg*) gene. The PCR amplification was performed in a 25 µl reaction mixture with 2.5U of Takara TaKaRa rTaq polymerase. In addition, 1X buffer with 1.5 mM $MgCl_2$ and 200 µM of dNTPs (Takara, Japan) were used in the PCR reaction mix. 0.2µM of each forward primer (Gldt218F) and reverse primer (Gldt218R) (Eurofins, Luxembourg) (Table 3.1.), 100ng of template DNA, and nuclease-free water were used for PCR amplification. The set included both positive controls and negative/no template controls. The PCR conditions included a 3-minute initial denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds and annealing at 65°C for 30 seconds. The extension was at 72°C for 30 seconds, following final extension at 72°C for 7 minutes. The reaction was then kept at hold at 4°C. 1.5% agarose gel electrophoresis was used to separate the amplified PCR products, and a UV transilluminator was then used to view the results.

3.2.8.4. PCR detection of *Cyclospora cayetanensis*

A conventional nested PCR method was employed to identify the presence of *C. cayetanensis* in stool, targeting a partial fragment of the SSU rRNA gene. The primary PCR amplification was conducted in a 25 µl reaction mixture containing 2.5U of Takara TaKaRa rTaq polymerase. Additionally, 1X buffer with 1.5 mM MgCl2 and 200 µM of dNTPs (Takara, Japan) were included in the PCR reaction mix. For PCR amplification, 0.2µM of each forward primer (F1E) and reverse primer (R2B) (Eurofins, Luxembourg) (Table 3.1.), 100ng of template DNA, and nuclease-free water were utilized. The set encompassed both positive controls and negative/no template controls. The primary PCR conditions comprised a 3-minute initial denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 40 seconds, concluding with a final extension at 72°C for 7 minutes. The secondary PCR reaction mixture (25 µl) included all the aforementioned reagents with the primer pairs: F3E (inner forward) and R4B (inner reverse) (Table 3.1.). The secondary PCR conditions consisted of a 3-minute initial denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. The reaction underwent a final extension at 72°C for 7 minutes, followed by holding at 4°C. To visualize the results, 1.5% agarose gel electrophoresis was employed to separate the amplified PCR products, and the results were observed using a UV transilluminator.

Table 3.1: Used primers for detection of common enteric parasites and corresponding annealing temperature and amplicon sizes

Organism name	Target gene	Primer name	Primer sequence (5' to 3')	Annealing temp.	Product size
Entamoeba spp.	SSU rRNA	EntaF	GTAGGATGAAACTGCGGACG	50°C	317 bp
		EntaR	AAAGCTCCCTCTCCGATGTC		
Cryptosporidium spp.	HSP70	CrHSP70_F	GCTGGTGATACTCACTTGGG	55°C	324bp
		CrHSP70_R	TCTCTTGTCCATACCAGCA		
Giardia lamblia	Beta-giardin	Gldt218F	ATAACGACGCCATCGCGGCTCTCAGGAA	65°C	218 bp
		Gldt218R	TTTGTGAGCGCTTCTGTCGTGGC		
Cyclospora cayetanensis	SSU rRNA	F1E	TACCCAATGAAAACAGTT	53°C	294bp
		R2B	CAGGAGAAGCCAAGGTAG		
		F3E	CCTTCCGCGCTTCGCTGCGT	58°C	
		R4B	CGTCTTCAAACCCCCTACTG		

3.2.9. Purification of PCR product

Using a High Pure PCR template purification kit (Roche, Germany), the PCR products were gel purified following manufacturer's instruction. The purified PCR products were quantified and visualized in 1.5% agarose gel.

3.2.10. Sequencing PCR reaction

Purified PCR products of the appropriate size were subjected to sequencing PCR in both directions, using the corresponding primer pairs. The following protocol was used to set up sequencing PCR (10 µl): 1X BigDye sequencing buffer (Applied Biosystems, USA), 2 µl of BigDye reaction premix (RRmix), 1.6 pmol of primer, 50-60 ng of purified template DNA, and nuclease-free water for volume make up. The sequencing reaction was carried out under the following conditions: initial

heat denaturation at 96°C for 2 minutes, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, and then hold at 4°C. All PCR reactions were performed in an ABI 9700 Genetic Analyzer® Thermal Cycler (Applied Biosystems, USA).

3.2.11. Purification of Sequencing PCR reaction

The products of sequencing PCR were mixed with 5 µl of 3M sodium acetate (pH- 4.8), 110 µl of absolute ethanol and 35 µl of nuclease free water. The content was mixed by vortexing and placed in ice for 1 hour. Then the mixture was centrifuged at 13000g for 40minutes at 4°C. The supernatants were carefully pipetted out from each tube and the pellets were rinsed with 70% ethanol. The mixture was again centrifuged at 13000g for 40minutes at 4°C. The supernatant was carefully discarded and pellets were air dried. The dried pellets were resuspended in 10 µl of Hidi Formamide by vortexing vigorously for 1 minute. The sample was then briefly centrifuged and incubated at 96°C for 2 minutes in a thermal cycler. The samples were then snap chilled in ice for 10 minutes. Next, the samples were again vortexed and collected at the bottom by briefly centrifugation. Finally, the samples were run through the ABI 3730 PRISM® DNA Analyzer (Applied Biosystem, USA) for bidirectional Sanger-dideoxy sequencing.

3.2.12. Phylogenetic analysis

The FinchTV 1.4 sequence analysis program was used to analyze the sequencing data. To compare nucleotide sequences generated in this study with reference sequences retrieved from the NCBI GenBank database, the Basic Local Alignment Search Tool (BLAST) was used. MEGA 6.0 software was used to align generated DNA consensus sequences to appropriate reference sequences. To estimate the phylogenetic relationships among the identified positive samples, the maximum likelihood (ML) method was used. The evolutionary distances were calculated using

the Tamura Nei parameter model in MEGA 6.0. The bootstrap method with 100 pseudo-replicates was used to assess the robustness of the resulting trees, and only values greater than 50% were reported.

3.2.13. Statistical Analysis

We explored factors (clinical symptoms, coinfection status with other intestinal parasites) linked to a positive result. Data analysis was carried out using GraphPad Prism v.9.0.0 (CA, USA) software. The Fisher's exact test was utilized to compare the sociodemographic parameters of the individuals with their infection status. The tests were two-sided, and a p-value <0.05 was deemed significant.

3.3. Results

3.3.1. Sociodemographic features of the patients

In this surveillance study, a total of 6320 samples were screened for the presence of parasites, with 3329 from ID & BG Hospital and 2991 from B. C. Roy Hospital. The gender distribution among admitted patients revealed a male-to-female ratio of 1.22, indicating that males constituted up a slightly higher proportion of the study population. The study included patients ranging in age from 8 months to 78 years, with a median age of 3.0 years. The age group >0–5 years constituted the largest proportion (59.28%) of the surveyed population. Among the participants, 31% (n/N = 1961/6320) hailed from rural areas. The majority of the individuals (≈69%; n/n = 4359/6320) resided in densely populated urban areas.

3.3.2. Prevalence and seasonal trend of parasitic diarrhea in the study population

Diarrhea caused by various pathogen species, including bacteria, viruses and parasites, was documented. The prevalence of intestinal parasitic infections remained relatively stable over the

2.5-year period, with an overall positivity rate of 18.56% (1173/6320). The highest positivity rate was observed in 2019, reaching 20%. The epidemiological study revealed that the overall prevalence of diarrheal cases remained relatively steady throughout the year, exhibiting no significant seasonal variation. While the total number of diarrheal cases did not fluctuate considerably across different seasons, the incidence of parasitic diarrheal diseases displayed a pronounced seasonal trend (Fig 3.1). This observation suggests that environmental and climatic factors may selectively influence the transmission dynamics of enteric parasites, without necessarily affecting the overall burden of diarrheal illnesses due to other etiological agents. The highest incidence rates were observed during the monsoon season of each year (23-25%), suggesting a potential influence of climatic factors, such as increased precipitation, humidity, and temperature, on the transmission dynamics of enteric parasites associated with diarrheal illnesses. In contrast, the dry or winter season exhibited a substantial decrease in the percentage of positive cases (10-11%) compared to other seasons. This marked reduction in case numbers during the cooler and drier months may be attributable to environmental conditions that are less favorable for the survival, dispersal, and infectivity of parasitic agents responsible for causing diarrheal diseases. Table 3.2. presented a comprehensive quantitative analysis, evaluating the characteristics of total parasitic infections and their associations with various potential risk factors.

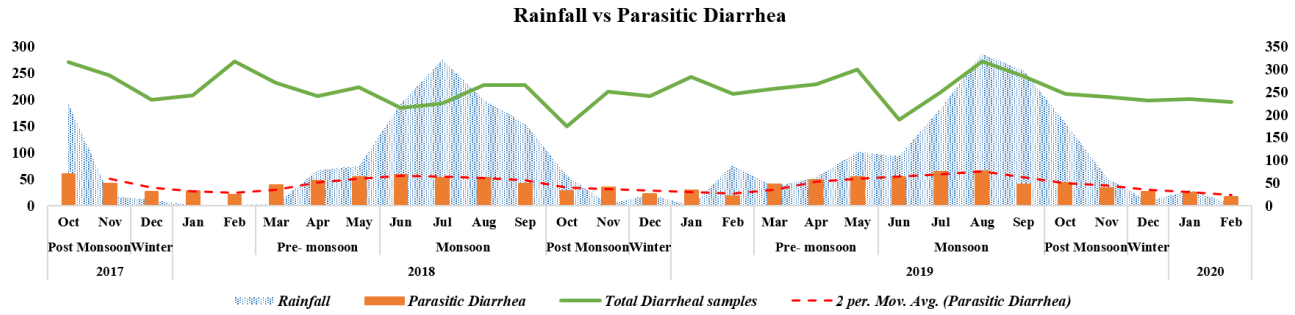


Fig 3.1. Seasonal distribution of parasitic infections in the study period (October, 2017-February, 2020). Month wise parasitic diarrheal vs rainfall (mm). Notable increase in infection during monsoon seasons was observed (Jun-Aug)

3.3.3. Prevalence and Features of *G. lamblia* infection

The prevalence of *G. lamblia*, as determined by conventional microscopic examination in this study, was 6.26% (n/N=396/6320; 95% CI: 5.69 - 6.89) (Fig 3.2 A. & B.). However, when polymerase chain reaction (PCR) screening was employed, a total of 422 positive samples were identified, including those initially detected through microscopy, resulting in an overall prevalence of 6.67% (n/N=422/6320, 95% CI 6.09- 7.32) (Fig 3.3.).

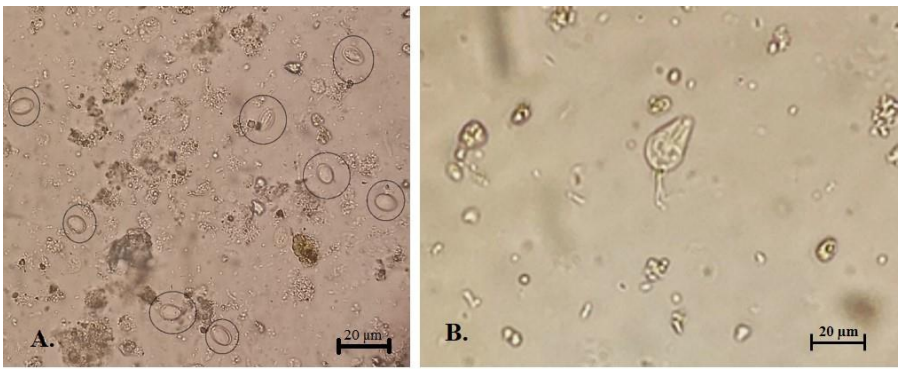


Fig 3.2.A. Cysts of *Giardia lamblia* in fecal sample of patients, wet mount [Iodine] (40x); **3.2.B.** Trophozoites of *Giardia lamblia* in fecal sample of patients, wet mount [Iodine] (40x)

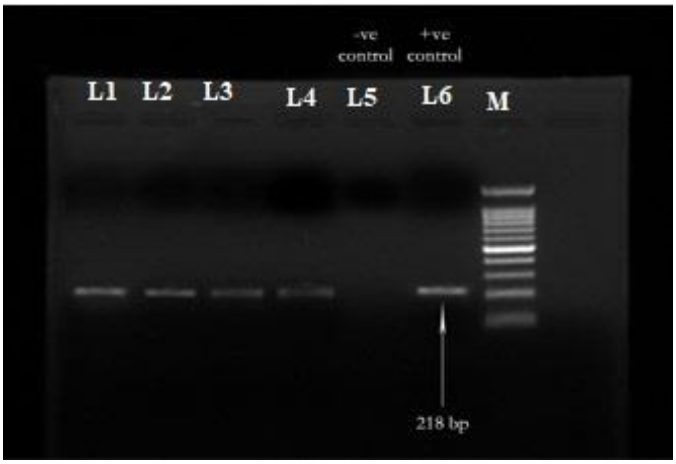


Fig 3.3. PCR product on agarose gel stained with ethidium bromide for *Giardia lamblia* detection from clinical samples (218bp). L1-4: Samples positive for *Giardia* (band obtained at 218bp, L5: Negative control, L6: Positive control, M: 50bp ladder marker

Stratified analysis revealed that *G. lamblia* infections were more prevalent among children aged between 0-5 years (8.21%, OR: 1.66, 95% CI: 1.16-2.38, P=0.004) and 5-12 years old (10.32%, OR: 2.14, 95% CI: 1.18-3.89, P=0.014), indicating a significantly higher risk of giardiasis in children under 12 years of age compared to other age groups (Table 3.2.). Conversely, the age group of 30-50 years exhibited a relatively lower risk of *G. lamblia* infection (2.16%, OR: 0.41, 95% CI: 0.23-0.72, P=0.002) compared to other age cohorts. The gender distribution analysis showed a slightly higher prevalence in males (6.7%) compared to females (6.64%), although this difference was not statistically significant (OR: 1.01, 95% CI: 0.82-1.23, P=0.956) (Table 3.2.). Notably, individuals residing in rural areas had significantly higher infection rates (10.24%, OR: 2.13, 95% CI: 1.75-2.61, P=0.0001) compared to their urban counterparts (Table 3.2.). *G. lamblia* maintains a relatively constant baseline incidence rate, suggesting an endemic nature of transmission within the population. However, during the monsoon post-monsoon season, a slight elevation in the number of new cases is typically observed, potentially attributable to increased rainfall facilitating the dispersal of infectious cysts through water sources favoring the survival

and spread of the parasite. Winter months generally witnessed periodic declines in giardiasis incidence (Fig 3.4.).

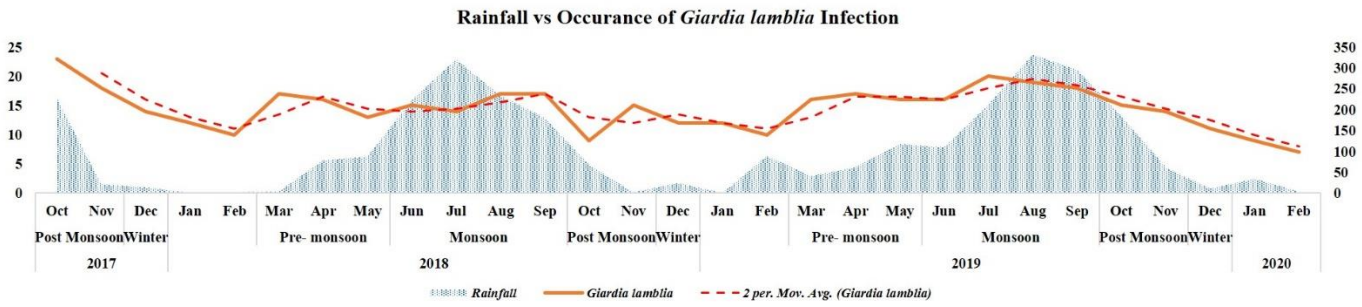


Fig 3.4. Seasonal distribution of *G. lamblia* infection in the study period (October, 2017-February, 2020). Month wise *G. lamblia* infection vs rainfall (mm). A steady rate of infection with a marginal rise in the monsoon season was observed.

Among the 422 confirmed cases of *G. lamblia* infection identified in the study, 41.31% (n/N=174/422, 95% CI 36.63-45.99) were classified as sole or mono-infections, where *G. lamblia* was the sole etiological agent detected. Conversely, the remaining 58.69% of cases were characterized by co-infections, wherein *G. lamblia* was found concurrently with other commonly encountered enteric pathogens. Notably, mixed infections involving *Giardia* were primarily associated with the presence of *Vibrio cholerae*, Rotavirus and *Escherichia coli* (Fig 3.5.). These co-infecting agents were frequently detected alongside *G. lamblia*, suggesting potential synergistic interactions or shared risk factors contributing to the acquisition and manifestation of these multiple enteric infections.

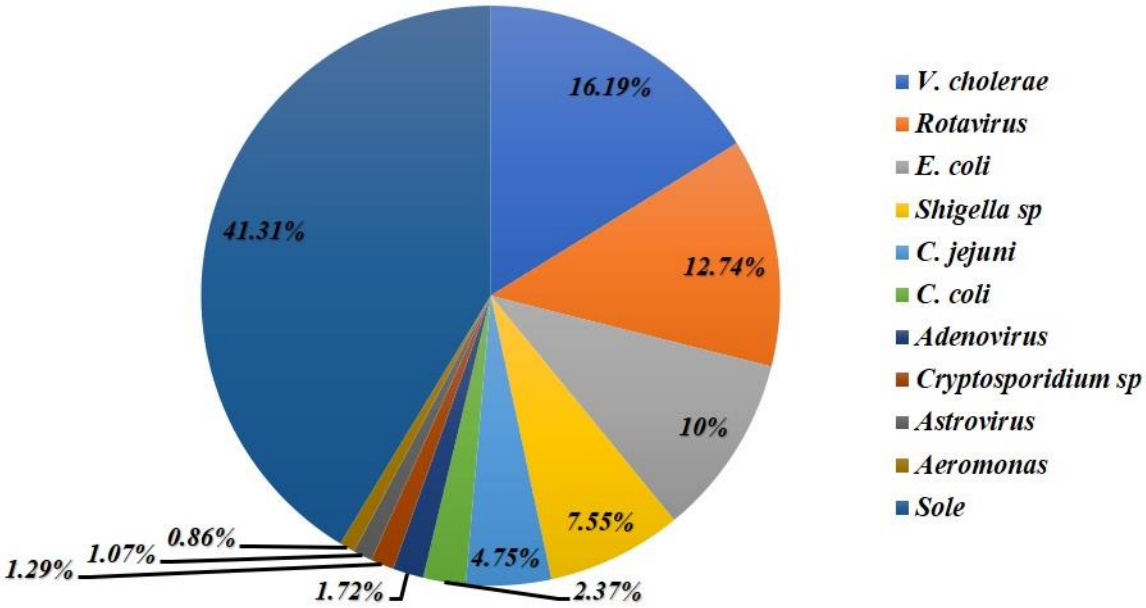


Fig 3.5. Coinfection status of *G. lamblia* positive cases

Out of the 6320 samples analyzed, the overall prevalence of *G. lamblia* infection was determined to be 6.67%. A notable spatial variation in the distribution of *G. lamblia* was observed across the study region (Fig 3.6.). The South 24 Parganas district exhibited a relatively high prevalence, exceeding 7%. In the metropolitan area of Kolkata, the prevalence rate surpassed 6%, while in the North 24 Parganas district, it was found to be above 5.5%. The remaining two districts, Howrah and Hugli, reported prevalence rates ranging between 4% and 5.5% (Table. 3.3.).

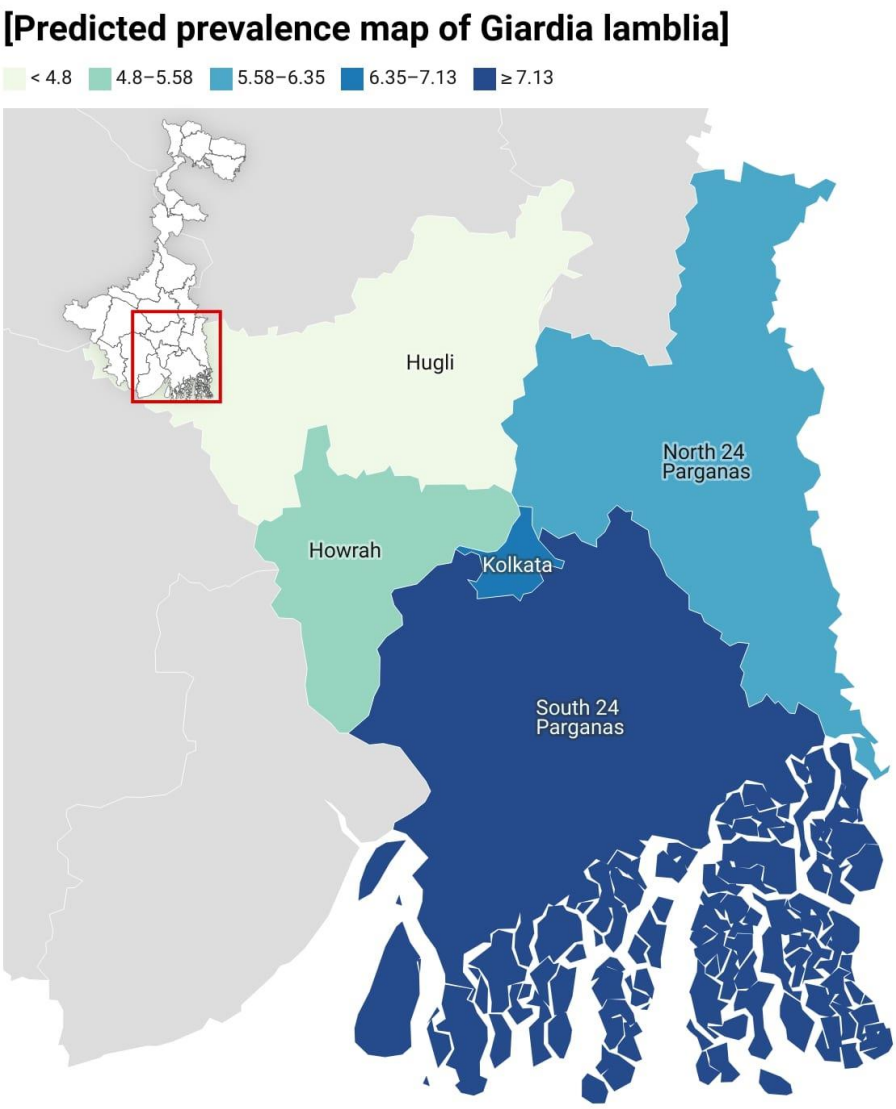


Fig 3.6. The spatial distribution and predicted prevalence of *Giardia lamblia* infection across the study region. The districts are color-coded according to their respective prevalence rates of this etiological agent. Darker shades represent areas with higher infection burdens, while lighter colors indicate regions with lower prevalence of *G. lamblia*

3.3.4. Prevalence and Features of *Entamoeba* spp. infection

The prevalence of *Entamoeba* spp., via conventional microscopic examination, was 4.01% (n/N=254/6320; 95% CI: 3.56 - 4.53) (Fig. 3.7. A. & B.). However, when PCR screening was employed, a total of 286 samples were identified as positive for *Entamoeba* spp., including those initially detected through microscopy. This resulted in an overall prevalence of 4.52% (n/N=286/6320, 95% CI: 4.04 - 5.07) (Fig 3.8.).

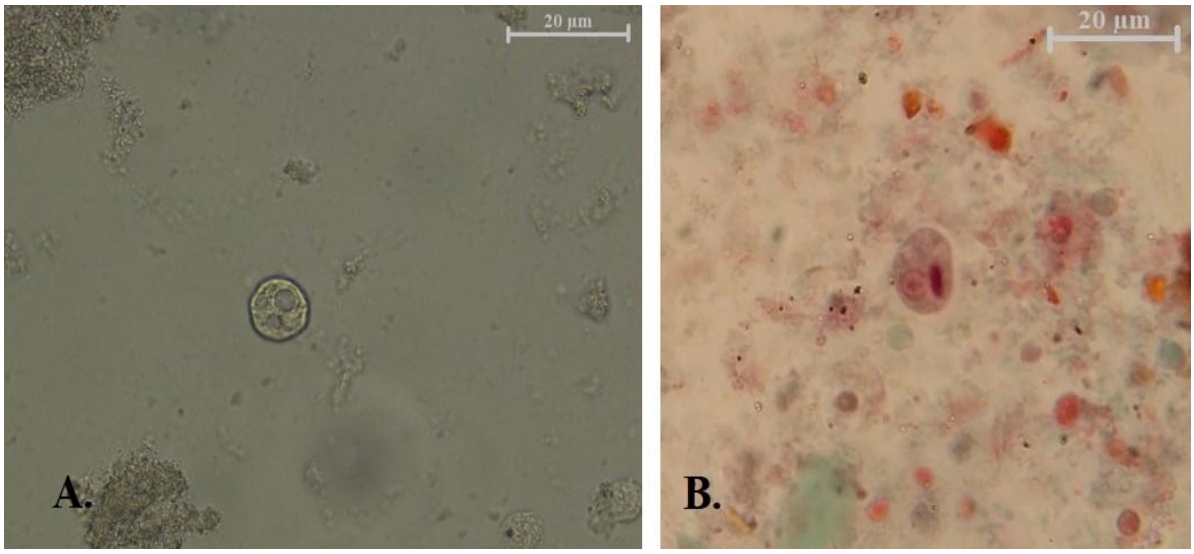


Fig 3.7.A. Cysts of *Entamoeba* spp. in fecal sample of patients, wet mount [Iodine] (40x); **3.7.B.** Trophozoites of *Entamoeba* spp. in fecal sample of patients, wet mount [Iodine] (40x)

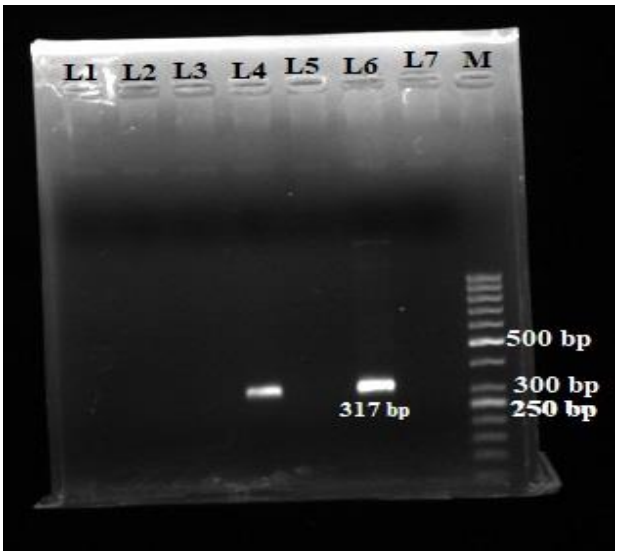


Fig 3.8. PCR product on agarose gel stained with ethidium bromide for all *Entamoeba* spp. detection from clinical samples (317bp). L 1-3, 5: *Entamoeba* negative samples; L4: Sample positive for *Entamoeba* (band obtained at 317bp); L6: Positive control; L7: Negative control; M: 50bp ladder marker

The statistical analysis revealed variations in the prevalence of *Entamoeba* spp. infections across different demographic groups. Children aged between 5-12 years exhibited a higher risk of infection (8.15%, OR: 1.65, 95% CI: 0.85-3.06, P=0.15) compared to other age groups. Conversely, individuals within the age range of 19-30 years demonstrated a relatively lower risk of *Entamoeba* infection (3.03%, OR: 0.77, 95% CI: 0.44-1.32, P=0.353). Regarding gender distribution, a slightly higher prevalence was observed among males (4.77%) compared to females (4.21%); however, this difference was not statistically significant (OR: 1.13, 95% CI: 0.89-1.44, P=0.301). Notably, individuals residing in urban areas exhibited significantly higher infection rates (5.32%, OR: 0.48, 95% CI: 0.36-0.66, P=0.0001) than their rural counterparts, potentially influenced by factors such as population density, sanitation infrastructure, and environmental conditions (Table 3.2.).

Surveillance data on the incidence of *Entamoeba* spp. did not reveal any discernible seasonal pattern or cyclical trend in transmission (Fig 3.9.). Instead, the observed prevalence exhibited abrupt fluctuations throughout the year, potentially indicative of transient carriage and intermittent spread of the infection, which could be attributed to factors such as compromised water sanitation or inadequate hygiene practices.

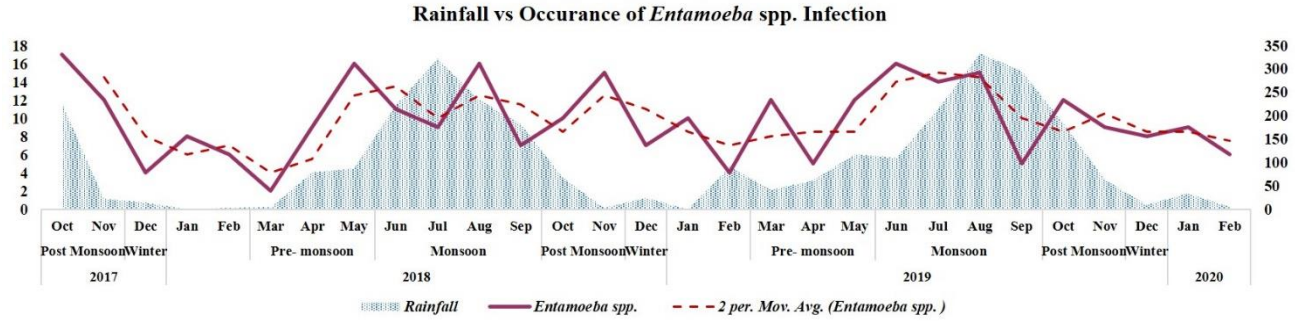


Fig 3.9. Seasonal distribution of *Entamoeba* infection in the study period (October, 2017-February, 2020). Month wise *Entamoeba* infection vs rainfall (mm). Abrupt fluctuations of infection rate was observed throughout the year.

Among the 286 confirmed cases of *Entamoeba* spp. infections identified in the study, 47.97% (n/N=137/286, 95% CI: 42.18-53.68) were classified as mono-infections, where at least one species of *Entamoeba* was the sole etiological agent detected. The remaining 52.03% of cases were characterized by co-infections, wherein *Entamoeba* spp. were found concurrently with other commonly encountered enteric pathogens. Notably, mixed infections involving *Entamoeba* spp. were primarily associated with the presence of *Vibrio parahaemolyticus* and Rotavirus (Fig 3.10.).

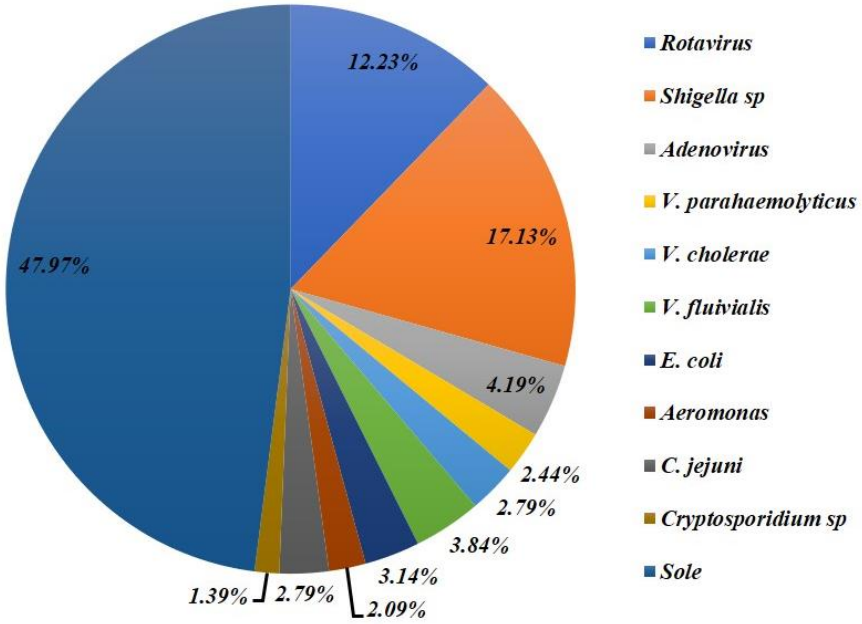


Fig 3.10. Coinfection status of *Entamoeba* spp. positive samples

Out of the 6320 samples analyzed, the overall prevalence of *Entamoeba* spp. was determined to be 4.52%. A notable spatial heterogeneity in the distribution of *Entamoeba* infections was observed across the study region (Fig 3.11.). Urban areas, including the city of Kolkata and its metropolitan surroundings, exhibited a relatively high prevalence, exceeding 5% of the examined cases. The South 24 Parganas and North 24 Parganas districts demonstrated a comparable prevalence, ranging from 4% to 4.5%. Conversely, the districts of Howrah and Hugli reported a lower prevalence, with less than 3.5% of cases attributed to *Entamoeba* spp. infections (Table 3.3.).

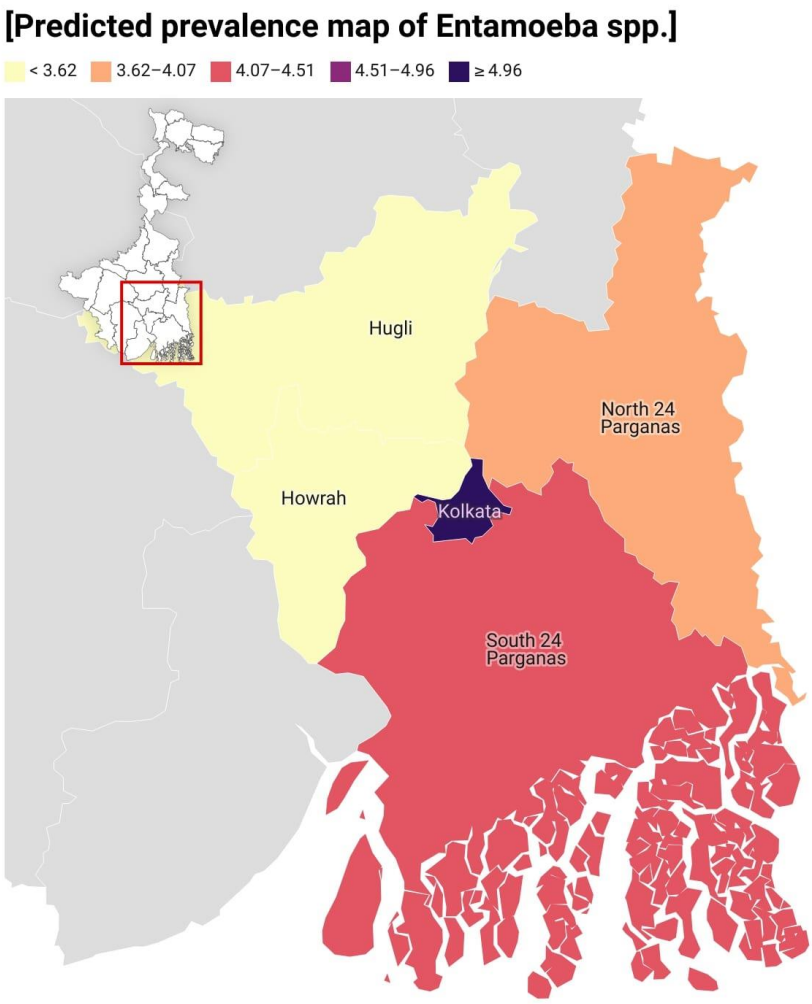


Fig 3.11. The spatial distribution and predicted prevalence of *Entamoeba* infection across the study region. The districts are color-coded according to their respective prevalence rates of this etiological agent. Darker shades represent areas with higher infection burdens, while lighter colors indicate regions with lower prevalence of *Entamoeba*

3.3.5. Prevalence and Features of *C. cayetanensis* infection

The prevalence of *Cyclospora cayetanensis*, was 2.83% (n/N=179/6320; 95% CI: 2.32- 3.12) via microscopy (Fig 3.12.A & B.). However, via PCR screening a total of 201 samples were identified positive for *C. cayetanensis*, including those initially detected through microscopy. This resulted in an overall prevalence of 3.18% (n/N=201/6320, 95% CI: 2.77 - 3.64) for this pathogen (Fig. 3.13.).

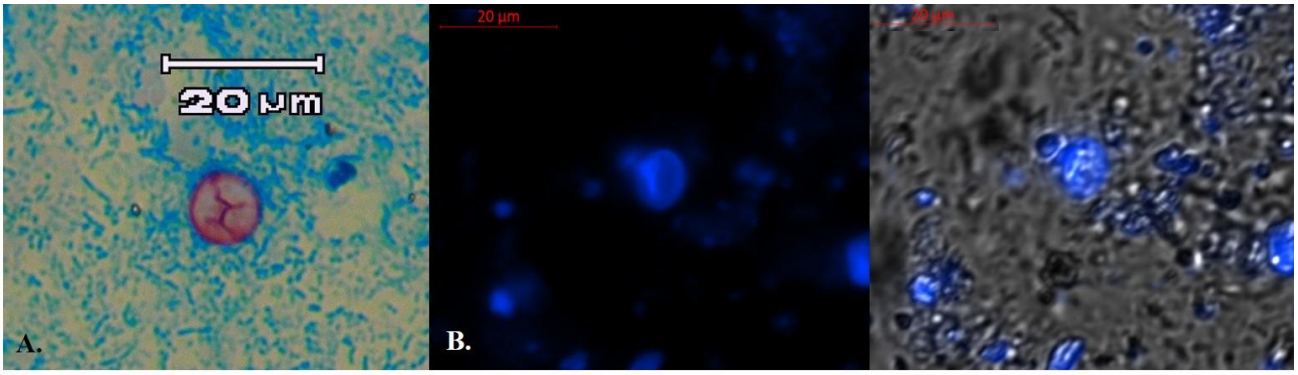


Fig 3.12.A. Oocysts of *C. cayetanensis* in fecal sample of patients, Kinyoun stained (100x); . **3.12.B.** Autofluorescence of *C. cayetanensis* oocysts observed with a 330–365 nm UV excitation filter (60x)

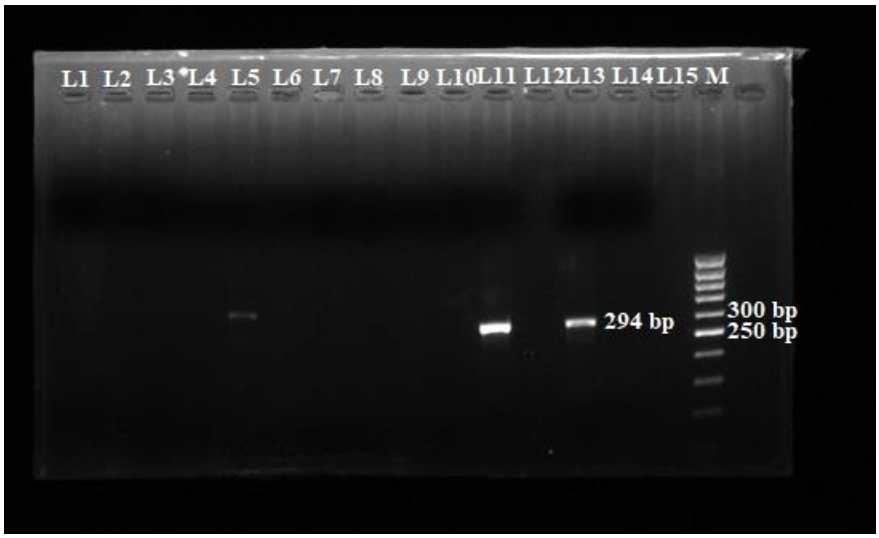


Fig 3.13. Amplified product of *C. cayetanensis* 18S rRNA on agarose gel(294bp). L1-4, L6-10: *Cyclospora* negative samples; L5 and L11: *Cyclospora* positive samples; L12: Negative control; L13: Positive control; M: 50bp marker

The surveillance study revealed variations in the prevalence of *Cyclospora cayetanensis* infections across different demographic groups. Children aged between 0-5 years exhibited a higher risk of infection (3.81%, OR: 1.51, 95% CI: 0.91-2.49, P=0.115) compared to other age groups, although this finding was not statistically significant. Conversely, individuals within the age range of 19-30 years demonstrated a relatively lower risk of *Cyclospora* infection (1.59%, OR: 0.61, 95% CI:

0.29-1.34, P=0.249), but this result was also not statistically significant. Regarding gender distribution, a slightly higher prevalence was observed among males (3.25%) compared to females (3.09%); however, this difference was not statistically significant (OR: 1.05, 95% CI: 0.79-1.39, P=0.773). Notably, individuals residing in rural areas exhibited significantly higher infection rates (4.94%, OR: 2.12, 95% CI: 1.60-2.81, P=0.0001) than their urban counterparts (Table 3.2.).

Report showed a pronounced peak in the incidence of *Cyclospora,* during the pre-monsoon and monsoon months spanning from March to August (Fig 3.14.), indicating a potentially significant influence of climatic and environmental factors on the transmission dynamics of this pathogen. This elevated incidence during the warmer and more humid months may be attributed to favorable conditions for the survival, dispersal, and infectivity of *Cyclospora* oocysts. Conversely, a drastic decline in cases was observed during the post-monsoon and winter months, suggesting that the cooler and drier months may impede the transmission cycle.

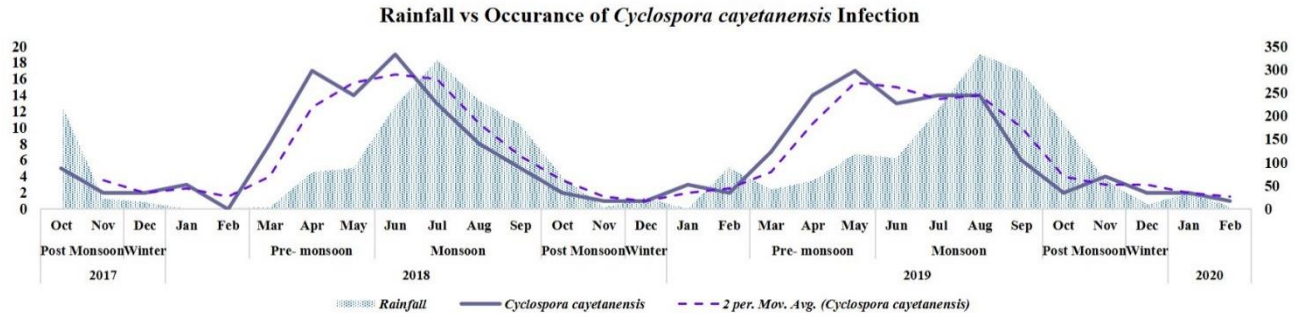


Fig 3.14. Seasonal distribution of *Cyclospora cayetanensis* infection in the study period (October, 2017-February, 2020). Month wise *C. cayetanensis* infection vs rainfall (mm). Notable increase in infection during premonsoon and monsoon season was observed (April-August)

Among the 201 confirmed cases of *Cyclospora cayetanensis* infections identified in the study, 51.76% (n/N=104/201, 95% CI: 44.87 - 58.55) were classified as mono-infections, where *C. cayetanensis* was the sole etiological agent detected. The remaining 48.24% of cases were

characterized by co-infections, wherein *Cyclospora* was found concurrently with other commonly encountered enteric pathogens. Notably, mixed infections involving *C. cayetanensis* were primarily associated with the presence of *Vibrio cholerae* and *Escherichia coli* (Fig. 3.15.).

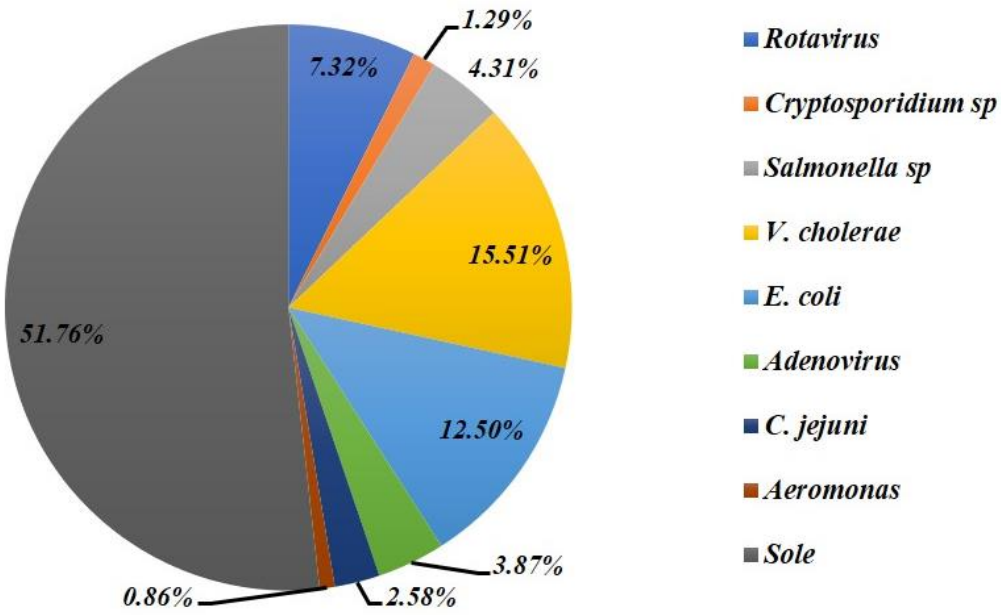


Fig 3.15. Coinfection status of *Cyclospora cayetanensis* positive samples

Out of the 6320 samples analyzed, the overall prevalence of *Cyclospora cayetanensis* was determined to be 3.18%. A notable spatial heterogeneity in the distribution of *Cyclospora* infections was observed across the study region (Fig. 3.16.). Both the South 24 Parganas and North 24 Parganas districts demonstrated a similar prevalence, exceeding 3.7%. The areas of Kolkata and Hugli also exhibited comparable prevalence rates for this pathogen, ranging between 2.1% and 2.6%. In contrast, the district of Howrah demonstrated a substantially lower prevalence of 1.58% (Table 3.3.).

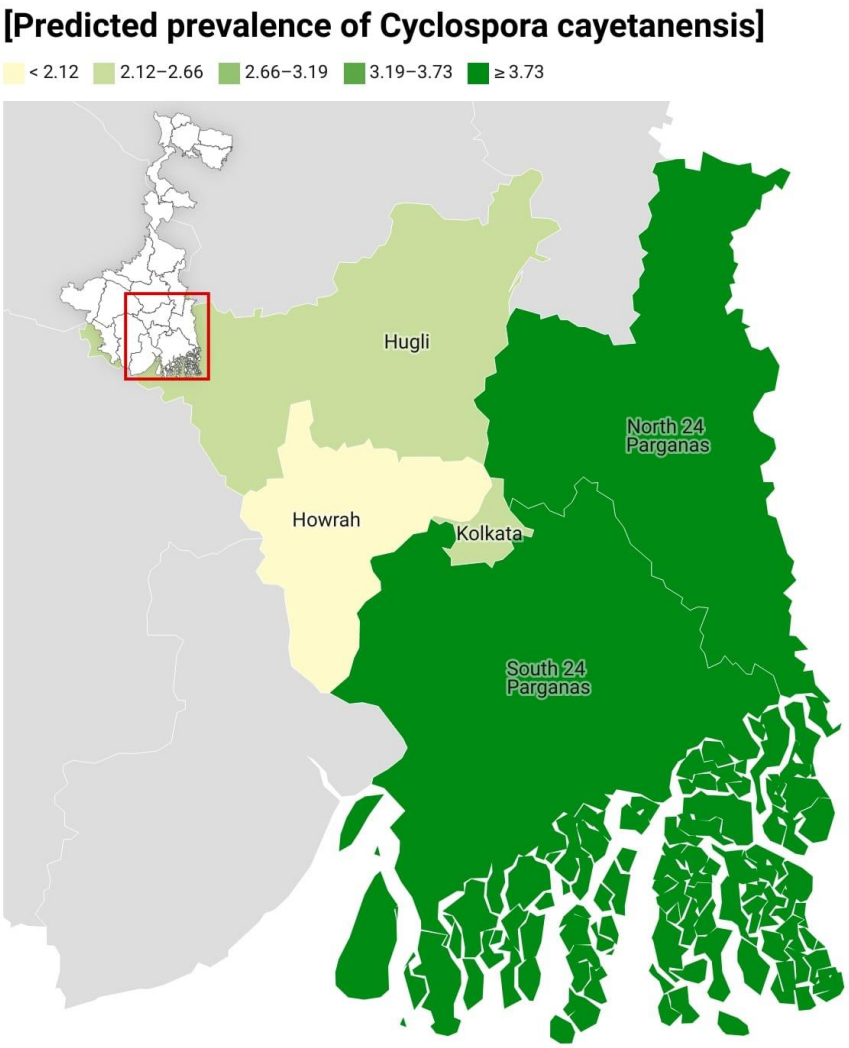


Fig 3.16. The spatial distribution and predicted prevalence of *C. cayetanensis* infection across the study region. The districts are color-coded according to their respective prevalence rates of this etiological agent. Darker shades represent areas with higher infection burdens, while lighter colors indicate regions with lower prevalence of *C. cayetanensis*

3.3.6. Prevalence and Features of *Cryptosporidium* spp. infection

The prevalence of *Cryptosporidium* spp., as determined by conventional microscopic examination, was 3.44% (n/N=218/6320; 95% CI: 3.03 - 3.93) (Fig. 3.17.). However, when PCR screening was employed, a total of 264 samples were identified as positive for *Cryptosporidium* spp., including

those initially detected through microscopy. This resulted in an overall prevalence of 4.17% (n/N=264/6320, 95% CI: 3.71 - 4.7) for this coccidian pathogen (Fig. 3.18.).

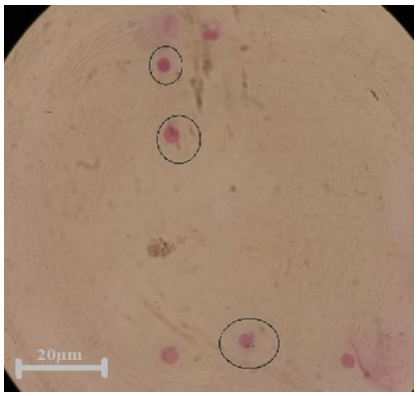


Fig 3.17. Oocysts of *Cryptosporidium* spp. in fecal sample of patients, Kinyoun stained (100x)

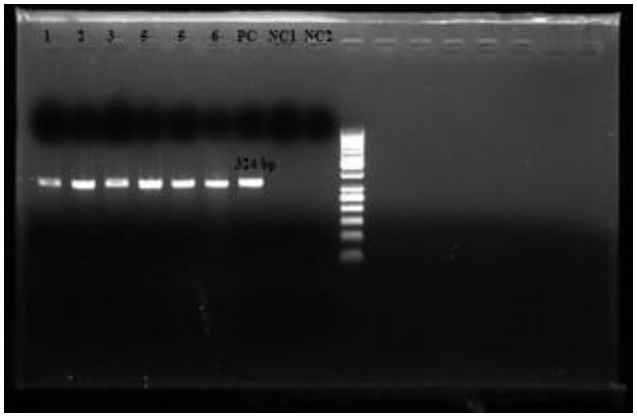


Fig 3.18. Amplified product of *Cryptosporidium* spp. Hsp70 on agarose gel(324bp). L1-6: *Cryptosporidium* positive samples; NC1-2: Negative control; PC: Positive control; M: 50bp marker

The study revealed variations in the prevalence of *Cryptosporidium* spp. infections across different demographic groups. Children aged between 0-5 years (5.04%, OR: 1.16, 95% CI: 0.78-1.76, P=0.497) and 5-12 years (5.43%, OR: 1.26, 95% CI: 0.62-2.65, P=0.55) exhibited a higher risk of *Cryptosporidium* infection compared to other age groups, although these findings were not statistically significant. In contrast, individuals within the age range of 19-30 years demonstrated a significantly lower risk of infection (1.27%, OR: 0.28, 95% CI: 0.13-0.60, P=0.001) compared

to other age cohorts. Regarding gender distribution, a slightly higher prevalence was observed among males (4.37%) compared to females (3.93%); however, this difference was not statistically significant (OR: 1.11, 95% CI: 0.87-1.43, P=0.411). Notably, similar to the trend observed with *Cyclospora* infections Individuals residing in rural areas exhibited significantly higher rates of *Cryptosporidium* infection (5.4%, OR: 1.35, 95% CI: 1.05-1.72, P=0.01) compared to their urban counterparts (Table 3.2.).

The epidemiological patterns of *Cryptosporidium* spp., another coccidian parasite, exhibited a similar seasonal trend to that of *Cyclospora*, with an escalation in the number of cases during the monsoon months and a substantial decline during the drier winter period (Fig 3.19.). This phenomenon suggests that climatic factors, such as increased precipitation and humidity, may create favorable conditions for the survival, transmission, and infectivity of coccidian parasites. The statistical analyses performed, as presented in Table 3.2., quantitatively assessed the occurrence of these parasitic infections and their association with seasonal variations.

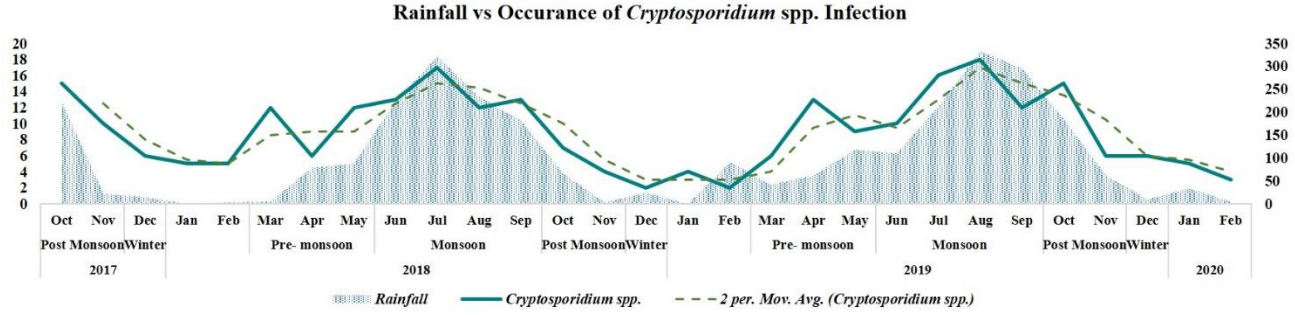


Fig 3.19. Seasonal distribution of *Cryptosporidium* spp. infection in the study period (October, 2017-February, 2020). Month wise *Cryptosporidium* infection vs rainfall (mm). Notable increase in infection during monsoon season was observed (Jun-Sept)

Among the 264 confirmed cases of *Cryptosporidium* spp. infections identified in the study, 40.21% (n/N=107/264, 95% CI: 34.42-46.17) were classified as mono-infections, where at least one species of *Cryptosporidium* was the sole etiological agent detected. Conversely, the remaining 59.79% of cases were characterized by co-infections, wherein this coccidian parasite was found concurrently with other commonly encountered enteric pathogens. Notably, mixed infections involving *Cryptosporidium* spp. were primarily associated with the presence of Rotavirus (Fig. 3.20.).

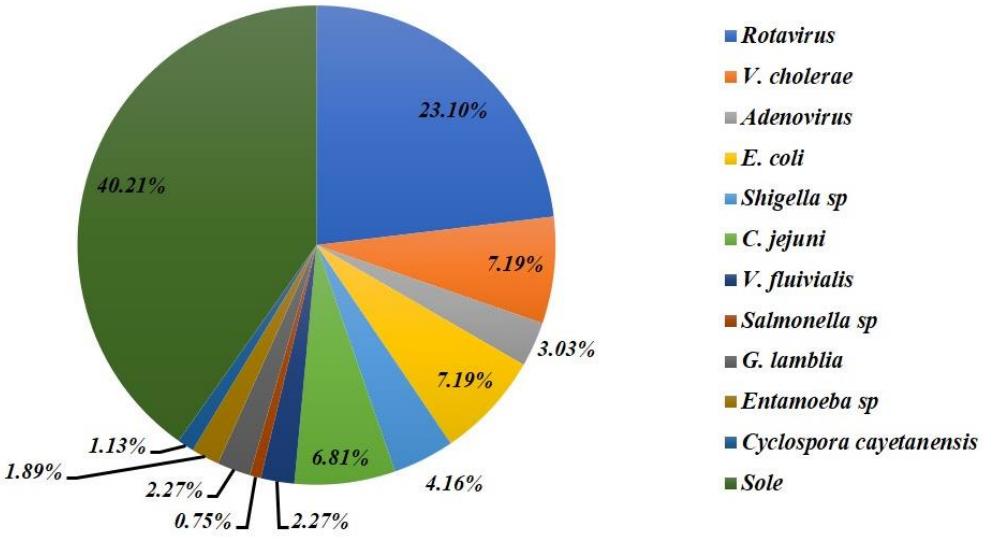


Fig 3.20. Coinfection status of *Cryptosporidium* spp. positive samples

Out of the 6320 samples analyzed, the overall prevalence of *Cryptosporidium* spp. was determined to be 4.17%. A notable spatial heterogeneity in the distribution of *Cryptosporidium* infections was observed across the study region (Fig. 3.21.). The South 24 Parganas district exhibited the highest prevalence rate of this coccidian parasite, exceeding 5% of the examined cases. This was followed by the Hugli district, where the prevalence ranged between 5.1% and 5.3%. The districts of

Howrah and Kolkata demonstrated similar prevalence rates, ranging from 3.7% to 5.1%. In contrast, the North 24 Parganas district displayed a relatively low prevalence of around 3% for *Cryptosporidium* infections (Table 3.3.).

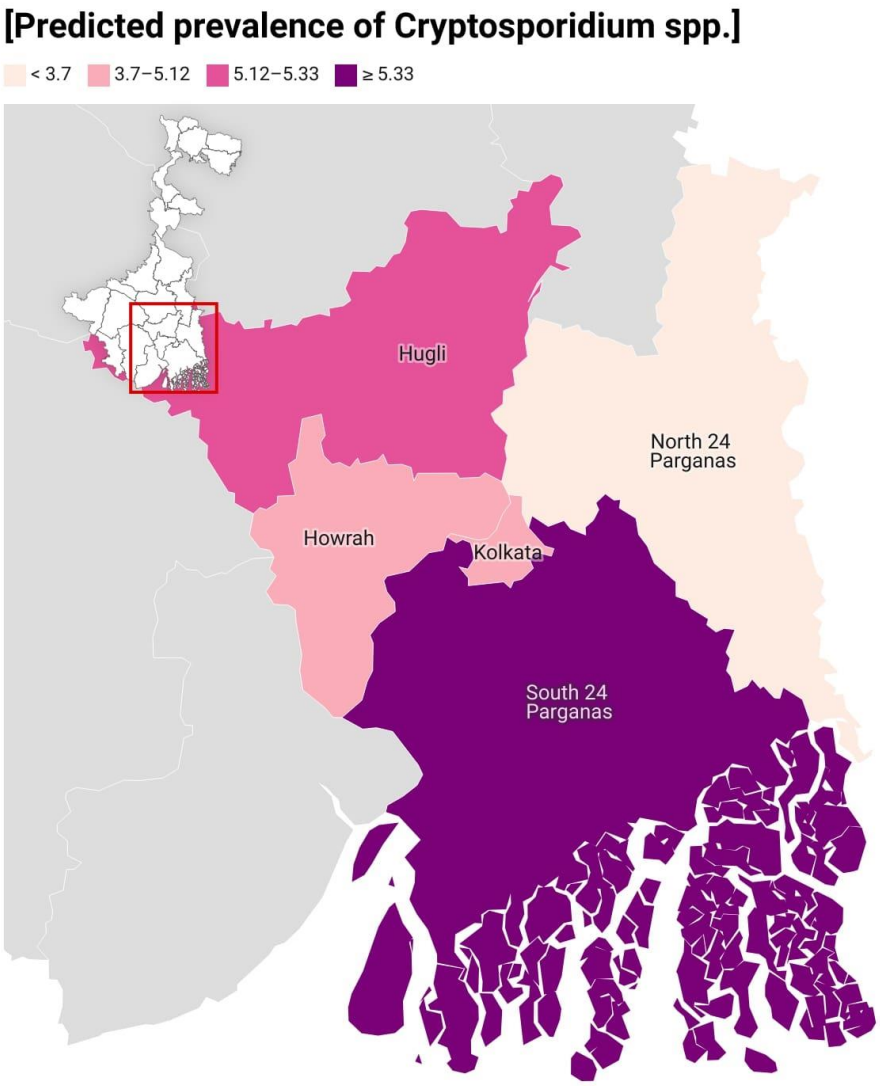


Fig 3.21. The spatial distribution and predicted prevalence of *Cryptosporidium* spp. infection across the study region. The districts are color-coded according to their respective prevalence rates of this etiological agent. Darker shades represent areas with higher infection burdens, while lighter colors indicate regions with lower prevalence of *Cryptosporidium* spp.

Table 3.2. Binary logistic regression models exploring risk factors for all parasitic infection (Value analyzed by Fisher's exact test is considered statically significant when $p < 0.05$)

Organism	Category	Group	% Positive	OR	95% CI	P value
Giardia lamblia	Gender	Male	6.7	1.01	0.82-1.23	0.956
		Female	6.64	-	-	-
	Age	0-5yr	8.21	1.66	1.16-2.38	0.004**
		5-12yr	10.32	2.14	1.18-3.89	0.014*
		12-19yr	6.94	1.38	0.72-2.56	0.307
		19-30yr	4.3	0.83	0.5-1.4	0.515
		30-50yr	2.16	0.41	0.23-0.72	0.002**
		>50yr	5.09	-	-	-
	Residential area	Rural	10.24	2.13	1.75-2.61	0.0001*
		Urban	5.07	-	-	-
	Seasonal prevalence	Pre monsoon	6.95	1.41	1.05-1.89	0.02*
		Monsoon	7.89	1.62	1.24-2.12	0.0004***
		Post monsoon	7.25	1.48	1.10-1.98	0.009**
		Winter	5.01	-	-	-
Entamoeba spp.	Gender	Male	4.77	1.13	0.89-1.44	0.301
		Female	4.21	-	-	-
	Age	0-5yr	4.21	0.81	0.56-1.20	0.303
		5-12yr	8.15	1.65	0.85-3.06	0.15
		12-19yr	5.09	0.99	0.49-1.94	0.99
		19-30yr	3.03	0.77	0.44-1.32	0.353
		30-50yr	4.89	0.95	0.60-1.52	0.906
		>50yr	5.09	-	-	-
	Residential area	Rural	2.75	0.48	0.36-0.66	0.0001*

		Urban	5.32	-	-	-
	Seasonal prevalence	Pre monsoon	4.09	1.29	0.901-1.87	0.183
		Monsoon	5.39	1.72	1.24-2.37	0.001**
		Post monsoon	5.78	1.85	1.32-2.62	0.0005***
		Winter	3.2	-	-	-
Cyclospora cayetanensis	Gender	Male	3.25	1.05	0.79-1.39	0.773
		Female	3.09	-	-	-
	Age	0-5yr	3.81	1.51	0.91-2.49	0.115
		5-12yr	2.17	0.84	0.30-2.51	0.99
		12-19yr	2.77	1.09	0.44-2.66	0.808
		19-30yr	1.59	0.61	0.29-1.34	0.249
		30-50yr	2.38	0.93	0.48-1.78	0.869
		>50yr	2.54	-	-	-
	Residential area	Rural	4.94	2.12	1.60-2.81	0.0001*
		Urban	2.38	-	-	-
	Seasonal prevalence	Pre monsoon	5.63	7.16	4.24-12.66	<0.0001*
		Monsoon	5.33	6.76	3.98-11.79	<0.0001*
		Post monsoon	1.23	1.49	0.77-2.91	0.278
		Winter	0.82	-	-	-
Cryptosporidium spp.	Gender	Male	4.37	1.11	0.87-1.43	0.411
		Female	3.93	-	-	-
	Age	0-5yr	5.04	1.16	0.78-1.76	0.497
		5-12yr	5.43	1.26	0.62-2.65	0.55
		12-19yr	1.84	0.41	0.15-1.15	0.101
		19-30yr	1.27	0.28	0.13-0.60	0.001**
		30-50yr	2.73	0.61	0.36-1.07	0.091

		>50yr	4.34	-	-	-
	Residential area	Rural	5.4	1.35	1.05-1.72	0.01*
		Urban	4.06	-	-	-
	Seasonal prevalence	Pre monsoon	8.12	4.41	3.04-6.49	<0.0001*
		Monsoon	3.36	1.73	1.15-2.61	0.009*
		Post monsoon	4.39	2.29	1.51-3.44	0.0001*
		Winter	1.96	-	-	-
All parasites	Gender	Male	19.1	1.08	0.95-1.23	0.2168
		Female	17.89	-	-	-
	Age	0-5yr	21.29	1.313	1.06-1.63	0.01*
		5-12yr	26.08	1.712	1.16-2.533	0.007*
		12-19yr	16.67	0.977	0.64-1.47	0.91
		19-30yr	11.17	0.609	0.44-0.84	0.002**
		30-50yr	12.17	0.6723	0.50-0.89	0.006**
		>50yr	17.09	-	-	-
	Residential area	Rural	23.35	1.5	1.32-1.71	0.0001*
		Urban	16.83	-	-	-
	Seasonal prevalence	Pre monsoon	20.93	2.14	1.76-2.59	0.0001*
		Monsoon	25.07	2.705	2.25-3.23	0.0001*
		Post monsoon	18.67	1.85	1.51-2.26	0.0001*
		Winter	11	-	-	-

Table 3.3. District-wise prevalence of all parasites

Organism	Surveyed Districts	Prevalence
Giardia lamblia	Kolkata	6.67%
	South 24 PGS	7.91%
	North 24 PGS	5.61%
	Howrah	5.11%
	Hugli	4.02%
Entamoeba spp.	Kolkata	5.41%
	South 24 PGS	4.17%
	North 24 PGS	4.01%
	Howrah	3.17%
	Hugli	3.29%
Cyclospora cayetanensis	Kolkata	2.55%
	South 24 PGS	4.27%
	North 24 PGS	3.78%
	Howrah	1.58%
	Hugli	2.19%
Cryptosporidium spp.	Kolkata	3.80%
	South 24 PGS	5.33%
	North 24 PGS	3.32%
	Howrah	3.70%
	Hugli	5.12%

3.3.7. Phylogenetic analysis and Assemblage distribution of *Giardia lamblia*:

The genetic diversity and population structure of *Giardia lamblia* were investigated through the analysis of the *beta-giardin (bg)* gene sequences obtained from 30 isolates. The results revealed the presence of both Assemblage A and Assemblage B genotypes within the study population. Specifically, 20% (n/N=6/30, 95% CI: 9.14-37.67) of the isolates (ISO-1, 9, 13, 17, 21, 25) were identified as belonging to the Subassemblage AII, while 13.33% (n/N=4/30, 95% CI: 4.7-30.3) of the isolates (ISO-4, 7, 19, 29) were classified as Subassemblage AI. Notably, the majority of the

isolates (n/N=20, 66.66%) belonged to Assemblage B, with 12 isolates (ISO-5, 6, 8, 11, 14, 16, 18, 22, 24, 26, 27, 30) (40%, n/N=12/30, 95% CI: 24.56-57.71) identified as Subassemblage BIII, and 8 isolates (ISO-2, 3, 10, 12, 15, 20, 23, 28) (26.67%, n/N=8/30, 95% CI: 13.98-44.65) classified as Subassemblage BIV. All isolates exhibited complete sequence identity with their respective reference strains, namely, AI-GL50803_4812, AII-DHA2_151428, BIII-KX085488, and BIV-GL50581_2741.The constructed phylogenetic tree (Fig. 3.22.) clearly delineated the 4 subassemblages of *G. lamblia* lineages into distinct clades, supported by high bootstrap values, indicating robust statistical support for their grouping. The observed genetic diversity within the *bg* gene highlights the presence of intraspecific variation and the circulation of multiple genotypes within the study population.

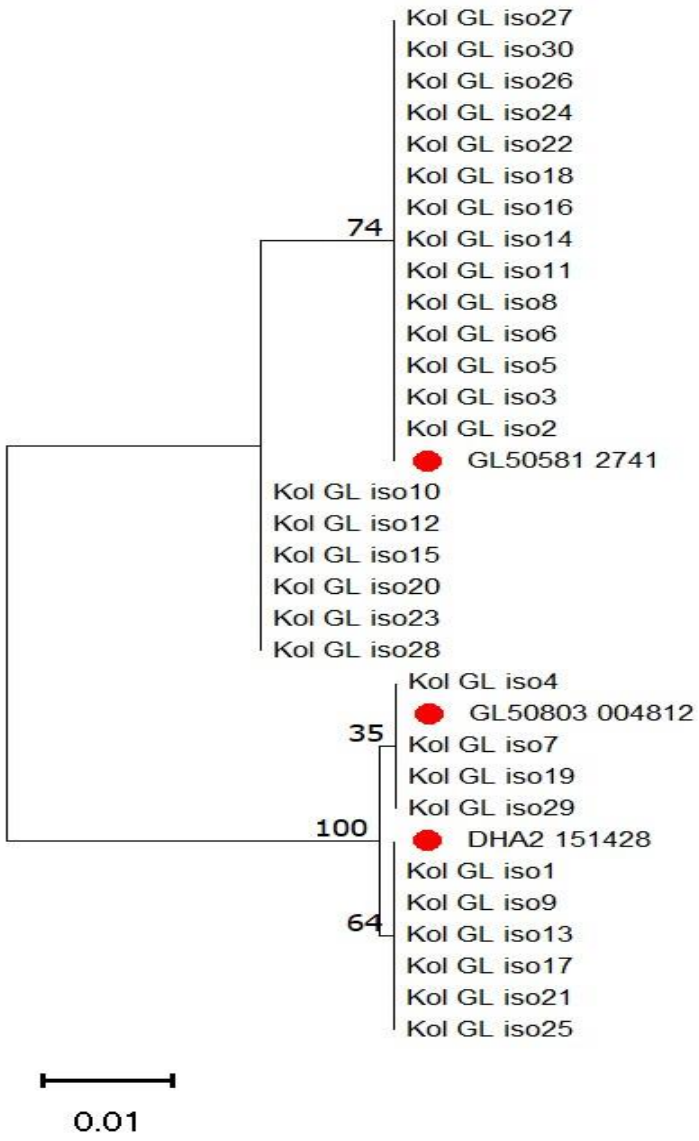


Fig 3.22. Phylogenetic analysis of *Giardia lamblia* isolates obtained in Kolkata and adjacent areas

3.3.8. Phylogenetic analysis and Species distribution of *Entamoeba* spp:

The genetic diversity and population structure of *Entamoeba* spp. were investigated through the analysis of the 18S rRNA locus sequences obtained from 30 isolates. The results revealed that 33.33% (n/N=10/30, 95% CI: 19.13-51.32) of the isolates (Iso-1, 2, 5, 8, 13, 18, 21, 22, 26, 29) were identified as *Entamoeba dispar*, while 20% (n/N=6/30, 95% CI: 9.14-37.67) belonged to the pathogenic species *Entamoeba histolytica* (Iso-6, 10, 14, 17, 20, 24). Notably, 46.67% (n/N=14/30, 95% CI: 29.83-64.18) of the isolates (Iso-3, 4, 7, 9, 11, 12, 15, 16, 19, 23, 25, 27, 28, 30) were identified as the relatively newly described species *Entamoeba moshkovskii*. Within the *E. dispar* population, three isolates (Iso-1, 22, 29) harbored a single nucleotide polymorphism (SNP) at position 123 G/C, while the remaining isolates exhibited complete sequence homology to the reference strain (Accession No. Z49256). In contrast, all *E. histolytica* isolates were identical to the reference sequence X56991, suggesting a lower level of genetic variation within this pathogenic species. The *E. moshkovskii* population exhibited some degree of genetic diversity, with three isolates (Iso-3, 11, 27) harboring two SNPs at positions 105 A/T and 356 A/T, and three isolates (Iso-23, 25, 30) exhibiting a single SNP at position 314 A/T. The remaining isolates were identical to the reference sequence AF149906.The constructed phylogenetic tree (Fig 3.23.) clearly delineated the three *Entamoeba* spp. lineages into distinct clades, supported by high bootstrap values, indicating robust statistical support for their grouping. The observed genetic variation within the partial 18S rRNA locus fragments highlights the presence of intraspecific diversity, particularly within the *E. moshkovskii* population.

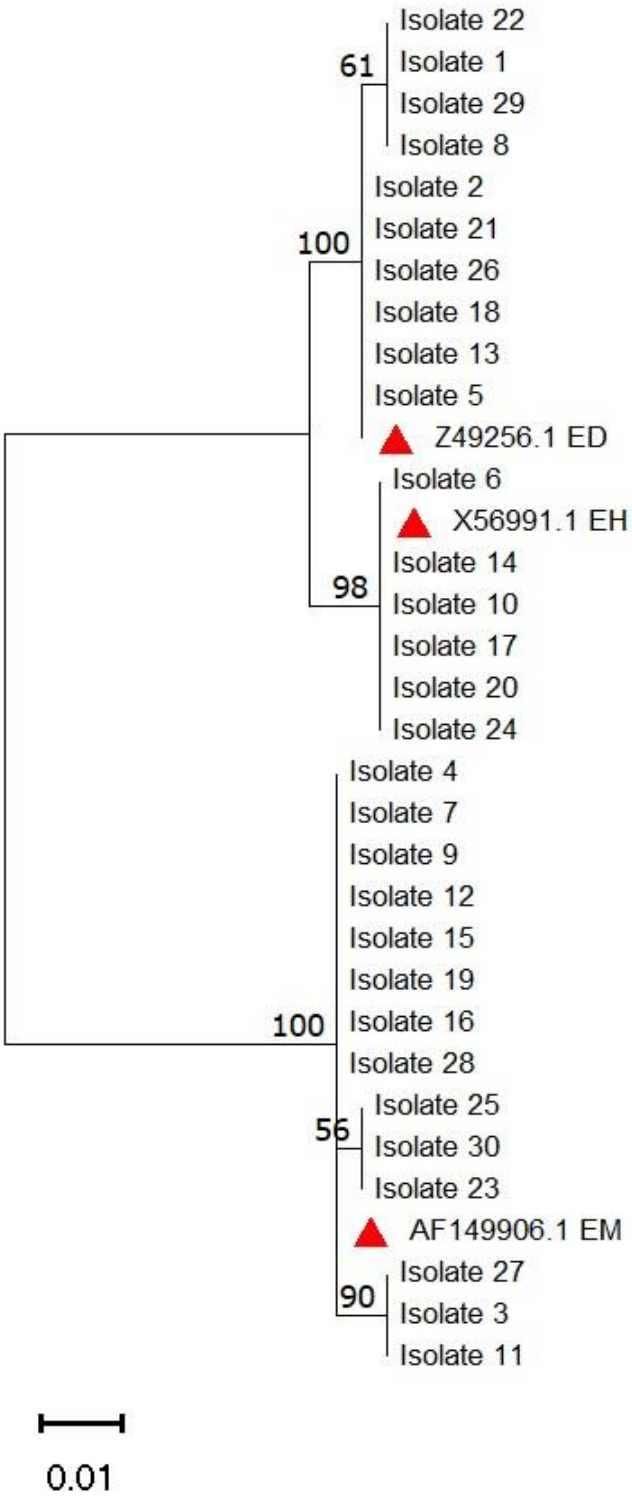


Fig 3.23. Phylogenetic analysis of *Entamoeba* spp. isolates obtained in Kolkata and adjacent areas

3.3.9. Phylogenetic relationship of *Cyclospora cayetanensis* isolates:

The genetic diversity of *Cyclospora cayetanensis* isolates was investigated by analyzing the 18S rRNA gene locus from 24 samples. The results revealed substantial heterogeneity at this partial locus, indicating a high degree of genetic variation within the study population. A total of 14 isolates (58.33%) exhibited complete sequence homology to the reference strains (Accession Nos. MN893894, KY770758, and KJ569534), while the remaining 10 isolates exhibited varying degrees of sequence polymorphisms, forming distinct clades. Four isolates (2, 5, 17, 24) harbored a single nucleotide polymorphism (SNP) at position 387 T/A, while three isolates (10, 13, 15) exhibited variations at sites 168 C/G and 255 T/G. Furthermore, two isolates (19 and 23) possessed three distinct SNPs at positions 140 C/G, 141 T/C, and 414 G/T when compared to the reference strains. The constructed phylogenetic tree (Fig. 3.24.) displayed well-resolved topologies for all the sequences obtained in this study, with the isolates clustering closely with both reference sequences and previously published sequences of the 18S rRNA gene. This phylogenetic analysis further corroborated the observed genetic diversity within the *C. cayetanensis* population, as evidenced by the formation of distinct clades supported by robust bootstrap values.

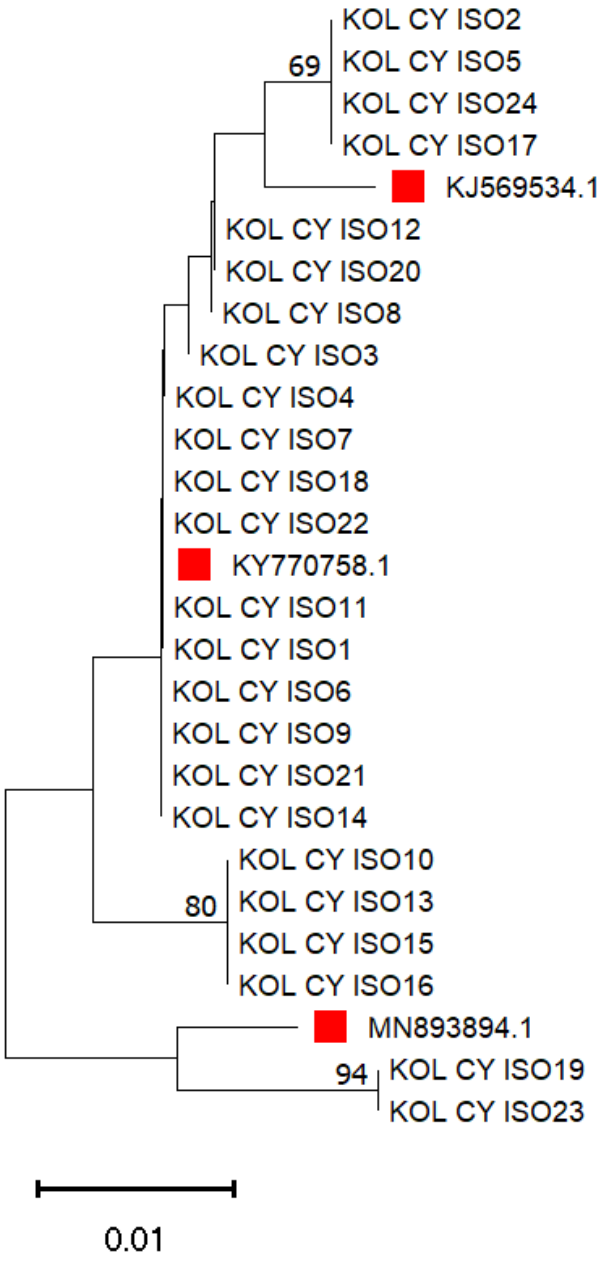


Fig 3.24. Phylogenetic analysis of *Cyclospora caytanensis* isolates obtained in Kolkata and adjacent areas.

3.3.10. Phylogenetic analysis and Species distribution of *Cryptosporidium* spp:

The genetic diversity and population structure of *Cryptosporidium* spp were investigated through the analysis of the Hsp70 gene sequences obtained from 30 isolates. The results revealed that 66.66% (n/N=20/30, 95% CI: 48.68-80.87) of the isolates were identified as *Cryptosporidium hominis*, while 30% (n/N=9/30, 95% CI: 16.52-48.02) belonged to the *Cryptosporidium parvum* species. Notably, one isolate (3.33%, n/N=1/30, 95% CI: 0.01-18.09) was identified as the relatively newly described species, *Cryptosporidium viatorum*, exhibiting complete sequence similarity to three previously reported *C. viatorum* isolates (GenBank Accession Nos. JX978274.1, JX978273.1, and JN846706.1).

Within the *C. hominis* population, three distinct clades were observed: 15 isolates (Iso 2, 5, 6, 8, 10, 11, 15, 16, 17, 18, 21, 22, 24, 26, 29) demonstrated complete sequence identity to the reference strains (GenBank Accession Nos. KX926461 and KM085025). Three isolates (Iso 9, 13, 27) harbored a single nucleotide polymorphism (SNP) at position 603T/G, while the remaining two isolates (Iso 4, 25) exhibited five distinct SNPs at positions 632T/C, 677C/G, 679T/G, 680C/A, and 881A/C, indicating a higher level of genetic variation within the *C. hominis* population. In contrast, the *C. parvum* population exhibited a relatively lower level of genetic diversity, with most isolates (Iso 1, 7, 14, 19, 20, 23, 28, 30) being identical to the reference sequence MK609845. Only one isolate (Iso 12) exhibited three distinct SNPs at positions 220C/T, 221A/C, and 268A/G. The constructed phylogenetic tree (Fig 3.25.) clearly delineated the three *Cryptosporidium* spp. lineages into distinct clades, supported by high bootstrap values, indicating robust statistical support for their grouping. The *C. viatorum* clade formed the most divergent lineage, with a bootstrap value of 100%, distinguishing it from the *C. hominis* and *C. parvum* clades.

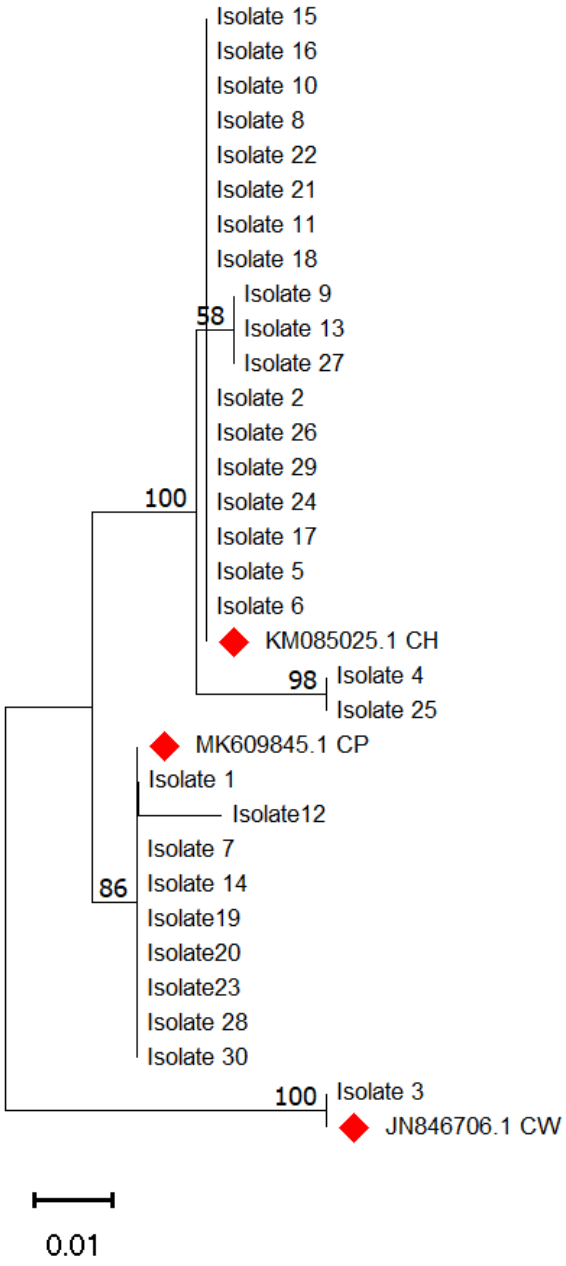


Fig 3.25. Phylogenetic analysis of *Cryptosporidium* spp. isolates obtained in Kolkata and adjacent areas

3.4. Discussion

In resource-limited settings, infectious intestinal diseases are often managed empirically without comprehensive diagnostic procedures or identification of the causative agents, as these conditions are frequently self-limiting. However, from a public health perspective, determining the specific pathogenic organism responsible for intestinal infections is crucial for several reasons: ensuring appropriate treatment, monitoring disease trends, enabling early outbreak detection, and informing the implementation of control measures and health policies. In this study, we conducted a comprehensive assessment to determine the current prevalence of *Giardia lamblia, Entamoeba* spp., *Cyclospora cayetanensis* and *Cryptosporidium* spp. in Kolkata and its neighboring districts. The detection methodology involved a combination of meticulous microscopic examination of stool samples and highly sensitive nucleic acid based molecular method i.e., PCR analysis. The application of PCR is strongly recommended, particularly when dealing with samples exhibiting low cyst loads, as it significantly enhances diagnostic sensitivity. Furthermore, in cases demanding accurate species identification, PCR becomes an indispensable tool due to the presence of morphologically indistinguishable species, especially within the genera *Entamoeba* and *Cryptosporidium*, which require specialized expertise for reliable microscopic identification. Our study highlighted the improved diagnostic accuracy achieved through PCR, as this molecular technique successfully detected parasite DNA in samples previously identified as negative by microscopic examination alone. Consequently, for optimal diagnostic accuracy, it is strongly recommended to employ a combined approach involving both microscopic examination and PCR (Sari et al., 2019). The integration of sensitive molecular techniques like PCR into diagnostic protocols is crucial for accurately identifying the causative agents of intestinal infections, enabling

targeted treatment, monitoring disease trends, and informing public health interventions and policies aimed at controlling the burden of these diseases.

This study found that *Giardia lamblia* infection rates were consistent throughout the year, with no significant seasonal variations. In this region, we found a significant decrease in the prevalence of giardiasis, which fell from 13% to 6.67% over the previous decade (Mukerjee et al., 2009). The observed decline in prevalence can be attributed in part to the Indian government's implementation of improved water supply and sanitation measures. These efforts may have contributed to the partial eradication of giardiasis. Despite ongoing efforts, the prevalence of giardiasis among children under 12 years old remains alarmingly high. Individuals who live in economically disadvantaged areas, including both rural and suburban Kolkata, have the highest infection rates. Giardiasis has been linked to poor growth and cognitive function in low- and middle-income countries, particularly among malnourished children under the age of five (Shaima et al., 2021). A study in Mexico found a link between giardiasis and vitamin A deficiency in school-aged children (Quihui-Cota et al., 2008). These findings highlight the negative effects of chronic *Giardia* infection on children's physical and mental development, which ultimately affects human resources and a country's overall economic health. Interestingly, the study conducted in the specified setting revealed a high prevalence of *Giardia*-related co-infections. This observation suggests that *G. lamblia* can efficiently colonize the host's intestinal tract without causing obvious symptoms. In this area, the prevalence of sole *Giardia* infections was lower, but co-infections with other enteric pathogens were more common. Numerous epidemiological studies conducted in endemic regions around the world consistently found no positive correlation between *Giardia* infections and the presence of diarrhea. These findings emphasize the prevalence of asymptomatic

giardiasis in certain environments, making it the norm rather than the exception (Bartelt et al., 2016; Koster et al., 2021).

The present study reported a significant prevalence of *Entamoeba* spp. in diarrhoeal stool samples from Kolkata, with more than half of these amoebic infections attributed to the newly identified species *E. moshkovskii*. This finding is concerning as it suggests a shift from the previously common *E. histolytica* to *E. moshkovskii*. Our data indicated that *Entamoeba* infections were most common in children aged 5–12 years. A recent study in Bangladesh found that 21% of children aged 2–5 years were infected with *Entamoeba* associated with diarrhoea (Ali et al., 2003). Other studies have also identified the three *Entamoeba* species as potential enteropathogens in patients with diarrhoea or dysentery (Ngui et al., 2012; Khairnar et al., 2007). Conducted among patients with diarrhoeal complaints, our study found a notable percentage of individuals infected with *E. moshkovskii*. The presence of mono-infection with *E. moshkovskii* was specifically associated with the occurrence of diarrhoea. Prevalence studies at I.D. Hospital and B.C. Roy Children Hospital highlighted that urban areas in Kolkata have a high burden of *Entamoeba* infections. Many positive samples also showed co-infections with bacteria, viruses, and other enteric parasites, consistent with findings from other studies (Sardar et al., 2023; Mukherjee et al., 2009). Unlike many parasitic infections, our survey did not observe any seasonal pattern for *Entamoeba* spp. infections.

Cyclospora cayetanensis is typically linked to traveler's diarrhea and is not usually included in laboratory diagnostics for immunocompetent patients, unless there is prolonged diarrhea or a history of travel to endemic regions. A community-based study in southern India reported a high burden of *C. cayetanensis* infection (22.2%) among low socioeconomic populations. Another study estimated the overall prevalence of *Cyclospora* in India to be between 5.05% and 6.11%, with an average prevalence of 5.58% (Li et al., 2020). In contrast, a hospital-based study found a

relatively low incidence of *C. cayetanensis* (1.1%) among immunocompromised and transplant recipients (Gupta, 2011). Our study identified a moderate prevalence of this coccidian parasite in the diarrheal population of Kolkata and the surrounding region. The prevalence of giardiasis among children under 5 years old is relatively high. Individuals who live in economically disadvantaged areas, in rural setting have the highest infection rate. Data from diverse human populations indicate that diarrhea significantly increases the risk of *C. cayetanensis* infection in both immunocompromised and immunocompetent individuals. Infection prevalence is notably higher among patients with diarrhea compared to those with other symptoms (Li et al., 2020). Most patients in this study exhibited acute diarrheal illness accompanied by abdominal cramps, which are classic symptoms of cyclosporiasis (Li et al., 2020; Sherchand et al., 2001; Martinez et al., 2002). Globally, *C. cayetanensis* infection follows a distinct seasonal pattern influenced by environmental factors such as rainfall, temperature, and humidity, which affect oocyst maturation and transmission dynamics. Incidence tends to rise during warm, rainy periods, as observed in countries like Guatemala, Honduras, Mexico, Jordan, Nepal, and China (Almeria et al., 2019). Our study noted a similar trend during the monsoon and pre-monsoon seasons (May to August), characterized by increased rainfall, humidity, and warm temperatures. The infection rate significantly declined during the drier winter months (November to February). An Indian study found that clinical cases peaked during summer, just before the onset of the rainy season, known as the pre-monsoon period (Tuli et al., 2008), a pattern also observed in Vietnam (Tram et al., 2008). In the majority of positive cases, *C. cayetanensis* was the sole pathogen responsible for gastrointestinal illness. In other samples, *Cyclospora* was present alongside another infectious agent, mainly bacteria. A hospital-based study among Mexican pediatric populations showed a higher prevalence of sole *Cyclospora* infections, followed by co-infections with other parasites

and helminths (Orozco-Mosqueda et al., 2014). Analysis of the 18S rRNA gene sequence of *C. cayetanensis* isolates from our study region revealed slight intraspecies variation, with one or two base differences. These findings align with a study in China, which also detected only minor sequence polymorphisms (Jiang et al., 2018; Zhou et al., 2011; Li et al., 2021). A study encompassing Mexico, Nepal, and Peru also reported minor genetic diversity within the species (Sulaiman et al., 2014). This study is the first to document the prevalence of *C. cayetanensis* among diarrheal patients in eastern India.

Our study of *Cryptosporidium* spp. infections in individuals with diarrhoea revealed distinct seasonal patterns, with a significant peak during the monsoon period and a smaller peak in the premonsoon season. This observation is consistent with a previous study (Das et al., 2006), who found similar patterns in children aged 0-5 years. A notable aspect of our study is the frequent co-infection of *Cryptosporidium* with diarrhoeagenic rotavirus. There are similar studies where *Cryptosporidium* spp. infections infection has coincided frequently with other diarrheagenic viruses like Astrovirus (Naif et al., 2020). *Cryptosporidium* spp. remains a major opportunistic infection in HIV patients (Ahmadpour et al. 2020). This may be influenced by weakened immune systems in HIV patients, children and the elderly. Our study, conducted in two government hospitals serving lower socioeconomic populations, suggests that poor nutrition compromises immune response (Maggini et al. 2018). Additionally, factors such as smoking and alcohol consumption further weaken the immune systems of the elderly (Sarkar et al. 2015; Nouri-Shirazi and Guinet 2003), potentially exacerbating the severity of these infections. DNA sequencing in our study identified *C. hominis* as the predominant species, followed by *C. parvum*, with no significant clinical symptom differences. We also identified a case of *C. viatorum*, a species first described in 2012 among travellers returning to Great Britain from the Indian subcontinent (Elwin

et al. 2012). Though less common, *C. viatorum* should not be overlooked. The oocysts of *C. viatorum* are morphologically similar to those of *C. hominis* and *C. parvum*, making microscopic differentiation challenging (Sardar et al., 2021). Therefore, molecular diagnostic techniques like PCR and DNA sequencing are recommended for accurate identification. *C. viatorum*, causing symptoms such as diarrhoea, abdominal pain, fever, and occasionally nausea, is an enteric parasite with a global distribution, found in patients who have traveled to regions including Kenya, Guatemala, and the Indian subcontinent (Lebbad et al. 2013). These findings underscore the importance of precise diagnostic methods to understand and manage this emerging pathogen in the region.

Of particular interest was the observation that *Giardia* emerged as the most prevalent enteric protozoan in the region under study, with a notably high incidence among children under 12 years of age, reaching a prevalence rate of up to 10%. This finding underscores the significant burden posed by this parasitic infection, particularly among the pediatric population, a vulnerable age group susceptible to adverse health outcomes associated with giardiasis. Given the substantial prevalence of *Giardia lamblia* in the region, the next phase of the study aims to conduct a comprehensive molecular characterization of the local isolates circulating within this endemic setting. Such in-depth analyses will involve genotyping and phylogenetic studies to delineate the population structure, genetic diversity, and potential transmission dynamics of the parasite within the study area.

3.5. Conclusion

In conclusion, this comprehensive study underscores the persistent and significant burden of parasitic diarrheal diseases among the population of Kolkata and its neighbouring districts. The

findings accentuate the pressing need for further research to elucidate the intricate transmission dynamics of these enteric parasites, as well as the development of robust diagnostic tools to prevent the emergence and spread of drug-resistant strains. Notably, the study identified the presence of relatively novel enteric parasites, such as *Entamoeba moshkovskii*, *Cryptosporidium viatorum*, and *Cyclospora cayetanensis*, which have not been previously reported in this region but exhibited substantial prevalence rates. This discovery raises significant concerns regarding the potential impact of these emerging pathogens on public health and highlights the importance of continuous epidemiological surveillance. Furthermore, these results emphasize the urgent necessity for public health authorities and policymakers to prioritize the implementation of comprehensive prevention and control strategies to combat parasitic infections effectively. The development and implementation of targeted interventions, informed by accurate diagnostic methods and an in-depth understanding of transmission patterns, are crucial in mitigating the burden of parasitic diarrheal diseases within these communities.

Chapter 4

GENOTYPING OF DIARRHEA CAUSING G*iardia lamblia* LOCAL ISOLATES

4.1. Introduction

Giardia lamblia (also known as *G. duodenalis* or *G. intestinalis*), the non-invasive intestinal protozoan, is commonly linked to diarrhea affecting humans and animals viz., livestock, and wildlife (Takumi et al., 2012). More than 200 million cases are annually reported, among which 2.5 million cases are from developing countries (Thompson, 2000; Squire & Ryan, 2017). Infection begins when ingested cysts transform into trophozoites in the duodenum after passing through the acidic stomach environment. Trophozoites replicate and adhere to the intestinal mucosal surface, leading to malabsorption and symptoms of diarrhea. The encounter with bile induces trophozoites to undergo a transformation into the resilient "cyst" form within the jejunum. Subsequently, the transmission cycle is completed via he fecal-oral route when these cysts are expelled into feces. (Adam 2001). In 2004, giardiasis became part of the Neglected Tropical Disease Initiative (NTDI) by the World Health Organization (WHO) due to its elevated prevalence in tropical regions and recurring infections, characterized by persistent cyst shedding for 6 months or more in certain cases. (Julio et al., 2012; Bartelt et al., 2013). The prevalence of giardiasis is estimated to be 2-5% in developed countries and increases upto 30% in developing countries (Wang et al., 2019). While the infection is found higher in pre-school and school-going children, older individuals and those with compromised immune systems can also exhibit symptoms (Stark et al., 2009). A report found prevalence of giardiasis to be 17% in HIV patients in the United States

(Hooshyar et al., 2019). Children affected by giardiasis may endure challenges such as malnutrition and consequently growth retardation accompanied with poor cognition (Stark et al., 2009; Wang et al., 2019). Molecular genotyping plays a pivotal role in identifying contamination sources during the epidemiological investigation of *G. lamblia* (Cacciò & Ryan, 2008). *G. lamblia* displays notable genetic diversity with minimal morphological variation, constituting a species complex. For *G. lamblia* there are eight distinct assemblages (A-H), among which assemblages A and B emerged as major human pathogens (Štrkolcová et al., 2016). Assemblages A and B having a broad host range are found in various mammalian species (Takumi et al., 2012). In contrast, the rest assemblages viz., C-H exhibit restricted host ranges and infrequently infect humans. (Cacciò & Sprong, 2009; Mirdha et al., 2014; Fantinatti et al., 2020). Highly discriminative loci in the genome are used for studying genetic variation to determine assemblages. Typically, partial sequences of more than one housekeeping genes are analyzed for *G. lamblia* subtyping. The loci of choice are namely glutamate dehydrogenase *(gdh*), triose phosphate isomerase (*tpi*), β-giardin (*bg*) and small subunit ribosomal ribonucleic acid (SSU rRNA). The virulence of assemblages A and B and their epidemiological properties remains unclear (Cacciò & Ryan, 2008). A cross-sectional study in India reported the exclusive occurrence of assemblage B (Figueiredo et al., 2020). In 2008, a year-long survey conducted in hospitals in Kolkata reported an approximately 13% prevalence of *Giardia* (Mukherjee et al., 2009). Despite the notable prevalence, there is limited information on the variations in virulence and clinical manifestations among genotypes of *Giardia.* Therefore, our

current investigation aims to examine the epidemiological distribution of genetically diverse *G. lamblia* isolates obtained from human clinical samples. Additionally, we seek to establish correlations between the clinical presentation and infection with different genotypes, while also exploring potential risk factors associated with *Giardia* infection.

4.2. Materials and Methods

4.2.1. DNA extraction

Genomic DNA was extracted from the fresh samples using QIAamp DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The eluted DNA was stored at -20^{0}C until use.

4.2.2.PCR detection and genotyping

Presence of *G. lamblia* in clinical samples was confirmed by a conventional PCR method targeting a 218-bp region of the beta giardin (*bg*) gene of the parasite. For the PCR reaction (25µl), approximate 100ng of template DNA, 1X buffer with 1.5mM $MgCl_2$ (TaKaRa, Japan), 200µM of dNTP (TaKaRa, Japan), 0.2µM of each forward (Gldt218F: 5'-ATAACGACGCCATCGCGGCTCTCAGGAA- 3') and reverse (Gldt218R: 5'-TTTGTGAGCGCTTCTGTCGTGGC- 3') primers (Eurofins, Luxembourg) (Mukherjee et al., 2009) and 2.5U of TaKaRa Taq (TaKaRa, Japan) were utilized with nuclease free water. The PCR condition involved initial denaturation step at 95°C for 3min, followed by 35 cycles of 95°C for 30s, 65°C for 30s, and 72°C for 30s, with a final extension step at 72°C for 7 min and kept for hold at 4°C. Next, the *G. lamblia* positive samples were taken to perform the multilocus genotyping, using partial fragments of the following three genes: a 511-bp region of beta-giardin (*bg*), a 531-bp region of triosephosphate isomerase (*tpi*) and a 432-bp region of glutamate dehydrogenase (*gdh*). Nested PCR protocols were followed to amplify these loci, involving two sets of primers

for each locus. All of the amplification reactions (50µl) consisted approximate 200ng of template DNA, 1X buffer with 1.5mM $MgCl_2$ (TaKaRa, Japan), 200µM of dNTP (TaKaRa, Japan), 0.2µM of each forward (G7F for *bg* locus, GltpiF1 for *tpi* locus and GlgdhF1 for *gdh* locus) and reverse (G759R for *bg* locus, GltpiR1 for *tpi* locus and GlgdhR1 for *gdh* locus) primers (Eurofins, Luxembourg) and 2.5U of TaKaRa ExTaq (TaKaRa, Japan) with proofreading activity, in the first round. In the nested PCR reactions (50µl), all the above-mentioned reagents were combined, including internal forward (Glbg511F2 for *bg* locus, Glbg531F2 for *tpi* locus and Glgdh432F2 for *gdh* locus) and reverse (Glbg511R2R for *bg* locus, Gltpi531R2 for *tpi* locus and Glgdh432R2 for *gdh* locus) primers. All the primer sequences used are provided in Table 4.1. The primary and nested primer sequences for *bg* and *tpi* loci were taken from previously described protocols (Lalle et al., 2005; Sulaiman et al., 2003), incorporating minor modifications for optimal amplification. The primers for amplification of *gdh* locus were designed from conserved regions of *G. lamblia* Assemblage A and B and optimized accordingly.

For the *bg* locus, the primary PCR reaction involved 95°C for 3min of initial heat denaturation, followed by 35 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 45 sec, with a final extension of 72°C for 7 min. The secondary PCR was initiated with heat activation step at 95°C for 3min, followed by 35 cycles of 95 °C for 30sec, 55°C for 30sec, 72°C for 35sec, and a final extension step at 72°C for 7 min.

The primary PCR for the *tpi* locus was performed as follows: an initial heat denaturation step at 95°C for 3 min, 35 cycles of 95 °C for 30 sec, 51°C for 30 sec, and 72°C for 45 sec, and a final extension step at 72 °C for 7 min. The secondary PCR condition was: a heat activation stage at 95°C for 3 min, then 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 35 seconds, and finally an extension step at 72°C for 7min.

For the *gdh* locus, the primary PCR started with a heat denaturation step at 95 ℃ for 3 min. This was followed by 35 cycles, consisting of denaturation at 95℃ for 30 sec, annealing at 53℃ for 30 sec, and extension at 72℃ for 40 seconds. A final extension step was performed at 72 ℃ for 7 min. Subsequently, the secondary PCR was carried out as follows: initial denaturation at 95℃ for 3 min. This was followed by 35 cycles of the following steps: denaturation at 95℃ for 30 seconds, annealing at 57℃ for 30 sec, and extension at 72℃ for 30 sec. Finally, an extension step was performed at 72℃ for duration of 7 min. All PCR runs comprised both negative (no DNA template) and positive (known *G. lamblia* genomic DNA) controls. Secondary PCR products were separated by 1.5% agarose gel electrophoresis and visualised after ethidium bromide staining.

The purified *bg*, *tpi,* and *gdh* fragments of correct size, were directly subjected to sequencing PCR in both directions, using the corresponding internal primer pairs. The protocol for setting up Sequencing PCR (10µl) was: 1X BigDye sequencing buffer (Applied Biosystem, USA), 2µl of ready BigDye reaction premix (RRmix), 1.6pmol of primer, 50-60ng of purified template DNA and nuclease free water for volume make up. The sequencing PCR was performed with the condition as follows: initial heat denaturation of 96℃ for 2 min, followed by 25 cycles of 96℃ for 10 sec, 50℃ for 5 sec, 60℃ for 4 min and then hold at 4℃. All of the PCR reactions were carried out in an ABI 9700 Genetic Analyer® Thermal Cycler (Applied Biosystem, USA).

The product of the sequencing PCR was purified with 3M sodium acetate (pH 4.8), absolute ethanol and nuclease free water and then mixed with Hidi Formamide. Sanger-dideoxy sequencing was performed bi-directionally with ABI 3730 PRISM® DNA Analyzer (Applied Biosystem, USA).

Table 4.1. Primers, target loci, and amplicon sizes obtained for *G. lamblia* genotyping

Locus	Primer Name	Oligonucleotide Sequence (5'-3')	Tm (°C)	Fragment Size (bp)
bg	G7F	AAGCCCGACGACCTCACCCGCAGTGC	75.4	753
	G759R	GAGGCCGCCCTGGATCTTCGAGACGAC	76	
	Glbg511F2	TGGAGAACGAGATCGAGGTCCG	65.8	511
	Glbg511R2	CTCGACGAGCTTCGTGTT	56.3	
tpi	GltpiF1	AAATTATGCCTGCTCGTCG	55	610
	GltpiR1	CAAACCTTCTCCGCAAACC	57.5	
	GltpiF2	CCCTTCATCGGCGGTAACTT	60.5	531
	GltpiR2	GTGGCCACCACGCCCGTGCC	70.7	
gdh	GlgdhF1	TGGATGGATGACGCTGGACG	62.5	600
	GlgdhR1	CCCATTGGAGTCCGAGAAGGT	63.2	
	GlgdhF2	CAGTACAACTCTGCTCTCGG	60.5	432
	GlgdhR2	GTTGTCCTTGCACATCTCC	57.5	

4.2.3. Sequence polymorphism analysis and phylogenetic tree construction

The analysis of the sequencing data was conducted using the FinchTV 1.4 sequence analysis program (https://finchtv.software.informer.com/1.4/). To determine the genotypes of *G. lamblia*, the obtained nucleotide sequences of each gene were aligned with reference sequences (X85958, AY072723, AY072724, L02120, U57897, M84604 and L40510 for assemblage A; AY072726, AY072727, AY072728, AF069561, AF069560, AF069059, L40508 for assemblage B) from NCBI GenBank using ClustalW in Mega6.0. To investigate the evolution of both assemblage MLGs (Multi-Locus Genotypes), the PHYLOViZ online tool, (http://www.phyloviz.net/) was used.

To carry out the phylogenetic analysis on the obtained sequences from the *bg*, *tpi* and *gdh* loci, the abovementioned reference sequences, were concatenated and utilized. The evolutionary relationships were determined using the maximum likelihood (ML) method. The Tamura Nei

parameter model in MEGA 6.0 was employed to calculate the evolutionary distances. To assess the robustness of the resulting trees, the bootstrap method with 100 pseudo-replicates was applied, and only values >50% were reported. The sequences generated in this study were deposited in GenBank under the Accession numbers OQ968734-OQ968785 (*bg*), OQ968682- OQ968733 (*tpi*) and OQ968630-OQ968681 (*gdh*).

4.2.3. Genetic diversity and haplotype network

DnaSP v5.0 was utilized to determine various genetic diversity measures from the aligned sequences, including variable sites (S), the number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π). Patient geographic locations were used to analyze the spatial distribution of *Giardia* isolates within the study area. To understand the relationship between the genotypes of *G. lamblia* from various regions surrounding Kolkata, a Median Joining haplotype network was constructed using PopART v1.7. The isolates were color-coded based on specific areas, allowing the inference of relationships with the sequenced data. Two neutrality tests (Fu's FS and Tajima's D) were carried out also with DnaSP v5.0 to assess the historical dynamics within the *Giardia* assemblages.

4.2.5. Statistical Analysis

The data analysis was conducted using GraphPad Prism v.9.0.0 (CA, USA) software. The Chi-squared test was employed to compare the socio-demographic parameters of the patients with their *G. lamblia* infection status. Statistical significance was determined at a threshold of $P < 0.05$. The probability of co-infection with other enteropathogens between assemblage A and B was measured by calculating odds ratio (OR) at 95% confidence interval (95% CI).

4.3. Results

4.3.1. *Giardia* Genotypes

Out of all PCR-positive samples, the amplification reaction successfully produced the anticipated amplicons for the *bg, tpi* and *gdh* loci in 52 examined isolates (Fig. 4.1, 4.2 and 4.3). Upon sequence analysis, it was determined that both human infecting assemblages i.e., assemblage A and assemblage B are circulating in the population. 16 isolates (30.8%) belonged to assemblage A, 33 isolates (63.5%) belonged to assemblage B, and 3 isolates (5.7%) exhibited mixed assemblages, containing both assemblages A and B. The distribution of assemblages differed significantly between cases of sole infection and mixed infections. The occurrence of the A assemblage was significantly associated with sole infections (OR: 13.6, 95% CI: 3.26–50.08, $p < 0.001$). Assemblage B was more frequently encountered (75%) in cases of mixed infections.

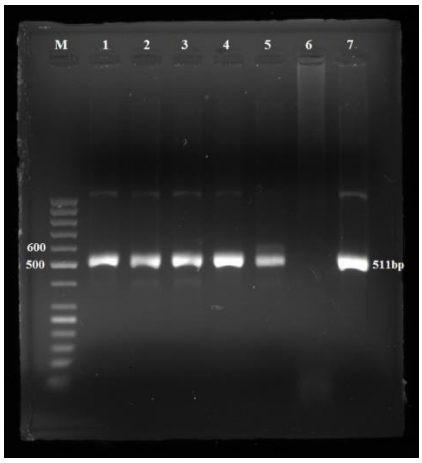


Fig. 4.1: Amplification of *Giardia lamblia* beta-giardin (*bg*) gene fragment (band obtained at 511bp). M: 50bp ladder marker, L 1-5: *Giardia* positive samples, L 6: negative control; L 7: positive control

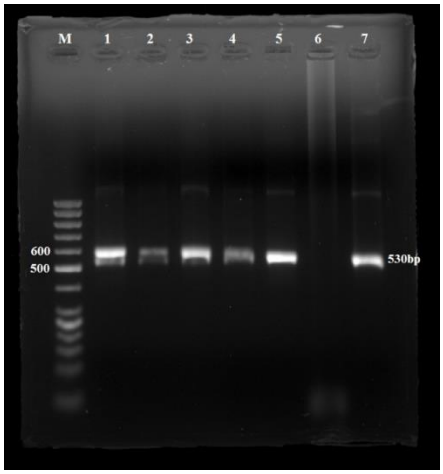


Fig. 4.2: Amplification of *Giardia lamblia* triosephosphate isomerase (*tpi*) gene fragment (band obtained at 530bp). M: 50bp ladder marker, L 1-5: *Giardia* positive samples, L 6: negative control; L 7: positive control

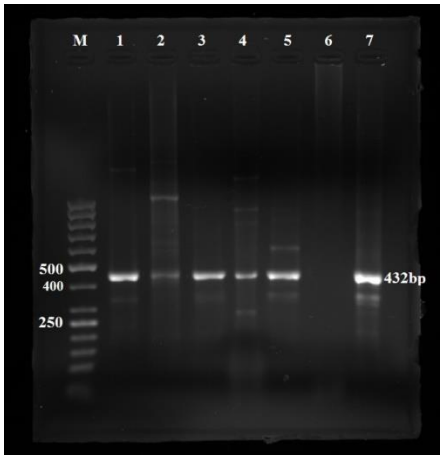


Fig 4.3: Amplification of *Giardia lamblia* glutamate dehydrogenase (*gdh*) gene fragment (band obtained at 432bp). M: 50bp ladder marker, L 1-5: *Giardia* positive samples, L 6: negative control; L 7: positive control

Among the 52 amplified isolates of *G. lamblia*, 16 (30.8%) were identified as belonging to assemblage A. Of the mixed infection cases, all three of them were identified as assemblage A at the *bg* locus. Out of these 19 isolates, 9 were assigned to the sub-assemblage AI-A1 at the *bg* locus and showed a 100% similarity to the reference sequence AI (X85958). 8 isolates belonged to the

sub-assemblage AII. Among them, 6 isolates exhibited a 100% similarity to the reference sequence AII-A2 (AY072723), while 2 isolates displayed polymorphisms at positions 472 (G>T) and 497 (C>T), respectively. From our analysis, we identified 2 isolates that belonged to subassemblage AII-A3. One of the isolates showed an exact match to the reference sequence (AY072724), while the other isolate had a single nucleotide polymorphism (SNP) at position 623 (C>T). Therefore, multiple sequence alignment of 52 samples at the *bg* locus revealed that 9 samples (17.3%) were classified as subassemblage AI whereas 10 (19.2%) were identified as AII type. From this specific locus, we identified a three novel sequences, one classified as AII-A3, along with two sequences categorized as Subtype AII-A2. The sequences were deposited in GenBank under accession no. OQ968767- OQ968785. The alignment analysis of the *tpi* locus revealed that 7 (13.5%) out of the 52 genotyped isolates were classified as sub-assemblage AI-A1 (6 were identical to reference: L02120) with 1 sample having a SNPs at position 972 (G>A). The remaining samples (9/52; 17.3%) were assigned to the sub-assemblage AII-A2 (U57897) and notably, two of them exhibited single nucleotide polymorphisms (SNPs) at positions 579 (C>T), 972 (G>A) and 630 (G>C), respectively. Based on the SNP analysis at this locus, we identified one novel sequence belonging to Subtype AII-A2 with another novel sequence from AI-A1 subtype. The obtained sequences were submitted to GenBank and assigned the accession numbers OQ968682- OQ968697. Out of the 52 samples analyzed, 18 isolates were identified as belonging to assemblage A at the *gdh* locus. 8 (15.4%) of them were classified as AI-A1. 6 of the 8 samples exhibited exact match to the reference sequence (M84604), while the remaining 2 samples displayed multiple single nucleotide polymorphisms (SNPs) at various positions along the sequence: Isolate 15 (OQ968635) - 311 (A/C), 437 (C/T), 544 (C/G); Isolate 27 (OQ968641) - 290 (C/A), 416 (A/C), 434 (A/G), 483 (T/G), 549 (G/T). Two novel isolates were encountered both of which belonged to Subtype AI-A1.

The accession numbers of the sequences are: OQ968630 - OQ968647. Out of the total examined isolates, 10 (19.2%) were classified as subassemblage AII-A2, and all of them demonstrated a 100% identity to the reference sequence (L40510).

Irrespective of the molecular marker utilized, the genetic diversity observed within assemblage B sequences was substantially greater than that found within assemblage A sequences. Out of the 52 samples analyzed, 33 isolates (63.5%) belonged to assemblage B with enormous genetic diversity. Multiple sequence alignments at the three loci involving identified numerous SNPs at multiple sites across the sequences compared to the reference sequences. The number of varying sites for each loci found are as follows: 13 at *gdh* locus, 15 at *tpi* locus and 17 at *bg* locus.

Upon analysis at *bg* locus, it was found that 33 (63.5%) out of 52 isolates belonged to assemblage B when initially aligned with reference sequences KJ188086.1 and KH926514.1. Among these, only 1 isolate (GlISO 46- OQ968762) demonstrated a perfect match (100% identity) with the reference sequence KJ188086. Additionally, 4 isolates displayed (GlISO 3- OQ968735, GlISO 12- OQ968741, GlISO 38- OQ968756 and GlISO 50- OQ968765) 1-3 single nucleotide polymorphisms (SNPs) at positions like 203 (C>G), 228 (A>G) and 427 (A>T) outside the sub-assemblage-defining positions (hotspot sites). 18 sequences exhibited a single SNP predominantly at position 354 (C>T). Specifically, GlISO33 (OQ968752) and GlISO45 (OQ968761) displayed a complete 100% identity with the reference sequence (AY647266). The remaining 6 isolates exhibited 1-4 SNPs at various positions. Two (GlISO35- OQ968754 and GlISO48- OQ968763) out of the 11 isolates were categorized as a novel subtype, with 1-3 SNPs at positions 172 (A>G), 324 (T>C) and 384 (C>T). Next, GlISO7 (OQ968738) displayed a SNP at the position 354 (T>C). From the analysis a total 7 novel sequences were obtained. All the sequences were deposited in GenBank under accession no. OQ968734 - OQ968766. When investigated at the *tpi* locus it was

found that 36 out of 52 isolates belonged to assemblage B. To analyze, the sequences were aligned with multiple reference sequences of B assemblage (AY368165.1, AF069561.1, GSB_93938, GL50801_1369, AF069560.1). Alignment analyses revealed that 22 isolates had a range of variations (1- 4 SNPs at places like: 162 G>A, 280 A>G, 304G>A, 354 G>A, 361G>T, 362G>A, 393 C>T, 504 C>T). On the other hand, GlISO 7 (OQ968702) had a SNP at position 39 (G>A) and GlISO 14 (OQ968706) had one SNP at position 39 (G>A) with another variation at position 362 G>A. 3 isolates exhibited an identical match with the reference sequences e.g., GSB_93938, GL50801_1369, AF069560.1 while 7 novel sequences were found in the region. The sequences could be found in NCBI GenBank under accession no. OQ968698 - OQ968733. Out of the 52 characterized isolates, 34 sequences belonged to assemblage B for *gdh* locus. When compared to the reference sequences AF069059.1 and JQ700429.1) 7 isolates showed a 100% identical match, indicating complete sequence similarity. On the other hand, 11 isolates exhibited 1-2 SNPs, resulting in a match ranging from 99.6% to 99.7% similarity. Two isolates (GlISO14- OQ968656 and GlISO 20- OQ968660) were found which possessed a SNP at a position 309 (T>C) and 10 isolates showed similarities with reference sequences i.e., GSB_21942, L40508.1, EU834843.1, and EU594666.1 having 1-2 SNPs at these three positions: 357 (T>C), 359 (C>T), 406 (T>C). Furthermore, four isolates (GlISO 7- OQ968652, GlISO 33- OQ968666, GlISO 51- OQ968680, GlISO 52- OQ968681) had the presence of 1 SNP at the hotspot sites (561 T>C, 612 A>G). Based on this locus,6 novel isolates were obtained.

4.3.2. MLG of the obtained Isolates

The analysis of multilocus genotypes (MLGs) in the 52 isolates uncovered extensive genetic diversity, with numerous heterogeneous positions observed within each gene fragment, particularly in the *bg* locus. We have encountered 3 mixed assemblage isolates (GlISO 26, GlISO

41, GlISO 52) in this study. To ensure accurate genotype assignment, three isolates of mixed assemblage were excluded from the analysis. A total of 10 MLGs (Fig. 4.4) were identified among the 16 successfully genotyped assemblage A samples at all three genetic loci (Table 4.3.). Among these, MLGs belonging to AII subtype was dominant, detected in 9 samples. The remaining MLGs, were all of AI subtype, found in 7 samples. No mixed subassemblages were detected within the A assemblage.

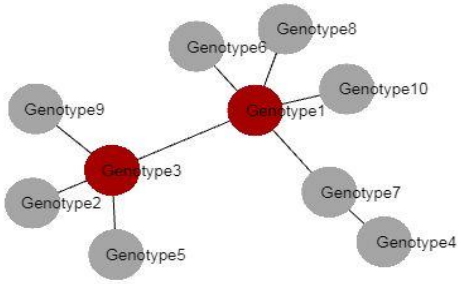


Fig. 4.4: Genetic relationship of multilocus genotypes (MLGs) of *Giardia lamblia* assemblage A based on eBURST analysis of *bg, tpi* and *gdh* concatenated sequences Each MLG is represented by a node. The primary founder of the group is colored maroon, while the subgroups are colored gray.

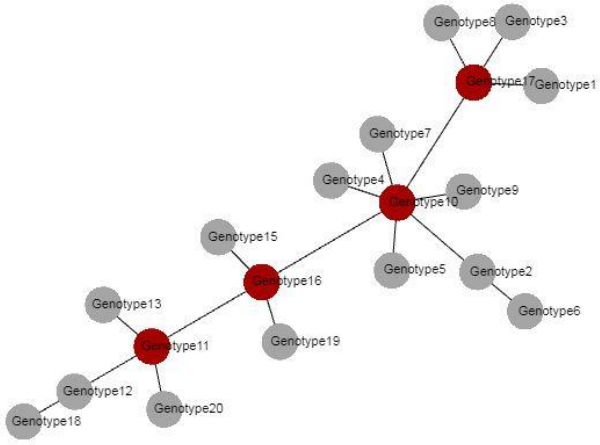


Fig. 4.5: Genetic relationship of multilocus genotypes (MLGs) of *Giardia lamblia* assemblage B based on eBURST analysis of *bg, tpi* and *gdh* concatenated sequences Each MLG is represented by a node. The primary founder of the group is colored maroon, while the subgroups are colored gray.

Among the 33 successfully genotyped assemblage B samples at all three genetic loci (Table 4.2.) a total of 26 MLGs (Fig. 4.5) were detected. The dominant MLG was MLG-B10, observed in 4 samples. MLG-B14, MLG-B11 were detected 3 and 2 samples respectively. The remaining MLGs were each detected in only one sample.

Table 4.2. Multilocus genotyping result and coinfection status of the 52 *Giardia*-positive samples

Isolate	*bg*	*tpi*	*gdh*	MLG *(bg+tpi+gdh)*	*Coinfection status*	*Other enteric pathogens*	*Patient Age (years)*
1	B	B	B	B	Coinfection	*V. cholerae*	26
2	AII_A2	AII_A2	AII_A2	AII	Sole	-	1.06
3	B	B	B	B	Coinfection	*V. cholerae + E. coli*	21
4	AI_A1	AI_A1*	AI_A1	AI	Sole	-	24
5	B	B	B	B	Coinfection	*S. flexneri*	1.07
6	B	B	B	B	Sole	-	21
7	B	B	B	B	Coinfection	*E. coli*	1.07
8	B	B	B	B	Coinfection	Adenovirus	1.09
9	AI_A1	AI_A1	AI_A1	AI	Coinfection	Rotavirus	11
10	B	B	B	B	Sole	-	0.08
11	AII_A2	AII_A2	AII_A2	AII	Sole	-	9
12	B	B	B	B	Coinfection	*Cryptosporidium sp.*	1.03
13	AII_A3*	AII_A2	AII_A2	AII	Sole	-	40
14	B	B	B	B	Sole	-	1.09
15	AI_A1	AI_A1	AI_A1*	AI	Sole	-	5.06
16	B	B	B	B	Coinfection	Rotavirus	1.08
17	B	B	B	B	Coinfection	*V. cholerae*	72
18	AII_A2	AII_A2*	AII_A2	AII	Sole	-	2.03
19	B	B	B	B	Coinfection	Adenovirus	0.09
20	B	B	B	B	Coinfection	*E. coli*	1
21	B	B	B	B	Coinfection	*V. cholerae+ C. jejuni+ E. coli*	1.04
22	AII_A3	AII_A2	AII_A2	AII	Sole	-	13
23	B	B	B	B	Sole	-	1
24	AI_A1	AI_A1	AI_A1	AI	Sole	-	52
25	AII_A2*	AII_A2	AII_A2	AII	Coinfection	*V. fluvialis*	38
26	AI_A1	B	AI_A1	Mixed (AI+B)	Coinfection	*V. cholerae*	24
27	AI_A1	AI_A1	AI_A1*	AI	Sole	-	1.09
28	B	B	B	B	Sole	-	0.05
29	B	B	B	B	Coinfection	*C. jejuni*	2

30	AI_A1	AI_A1	AI_A1	AI	Sole	-	0.08
31	AII_A2	AII_A2	AII_A2	AII	Sole	-	46
32	B	B	B	B	Coinfection	*V. cholerae*	54
33	B	B	B	B	Coinfection	*V. cholerae*+ *C. coli*	26
34	B	B	B	B	Coinfection	Rotavirus+ Adenovirus	1.08
35	B	B	B	B	Coinfection	*Aeromonas sp.*	10
36	B	B	B	B	Coinfection	Rotavirus	0.1
37	AII_A2	AII_A2	AII_A2	AII	Sole	-	7
38	B	B	B	B	Coinfection	Adenovirus	0.06
39	B	B	B	B	Sole	-	2.06
40	AI_A1	AI_A1	AI_A1	AI	Coinfection	*V. cholerae*	21
41	AII_A2	B	AII_A2	Mixed (AII+B)	Sole	-	1.07
42	B	B	B	B	Coinfection	*S. sonnei* + *C. jejuni*	0.09
43	B	B	B	B	Sole	-	1. 06
44	B	B	B	B	Coinfection	*C. coli*	35
45	B	B	B	B	Coinfection	*V. cholerae*	80
46	B	B	B	B	Coinfection	Rotavirus	1.07
47	AII_A2*	AII_A2	AII_A2	AII	Sole	-	67
48	B	B	B	B	Coinfection	Astrovirus	4.03
49	B	B	B	B	Coinfection	*V. cholerae*	60
50	B	B	B	B	Coinfection	Rotavirus+ Adenovirus	1
51	B	B	B	B	Sole	-	0.07
52	AI_A1	B	B	Mixed (AI+B)	Coinfection	*E. coli*	2.03

4.3.3. **Phylogenetic analysis and evolutionary relationship**

Phylogenetic analyses conducted using the ML method demonstrated that all sequences obtained in this study, at the *bg, tpi* and *gdh* loci, showed well-resolved topologies presenting distinct clusters for A and B assemblages alongside the reference sequences and previously published sequences sourced from GenBank. A concatenated phylogenetic tree was constructed to gain insights into the relative positions of the isolates in relation to one another (Fig: 4.6). The mixed assemblage samples, namely GlISO 26, GlISO 41, and GlISO 52, formed a distinct cluster located in the middle of the phylogenetic tree. These findings support the accurate grouping and

alignment of the sequences within their respective genetic contexts. The analysis of the evolutionary relationship among subtypes within the A assemblage revealed that the genotypes 1 and 3 served as the primary founders, from which various subgroups were derived. In case of B assemblage, genotype 10 and 16 emerged as the primary founders, while genotype 11 and 17 acted as subgroup founders.

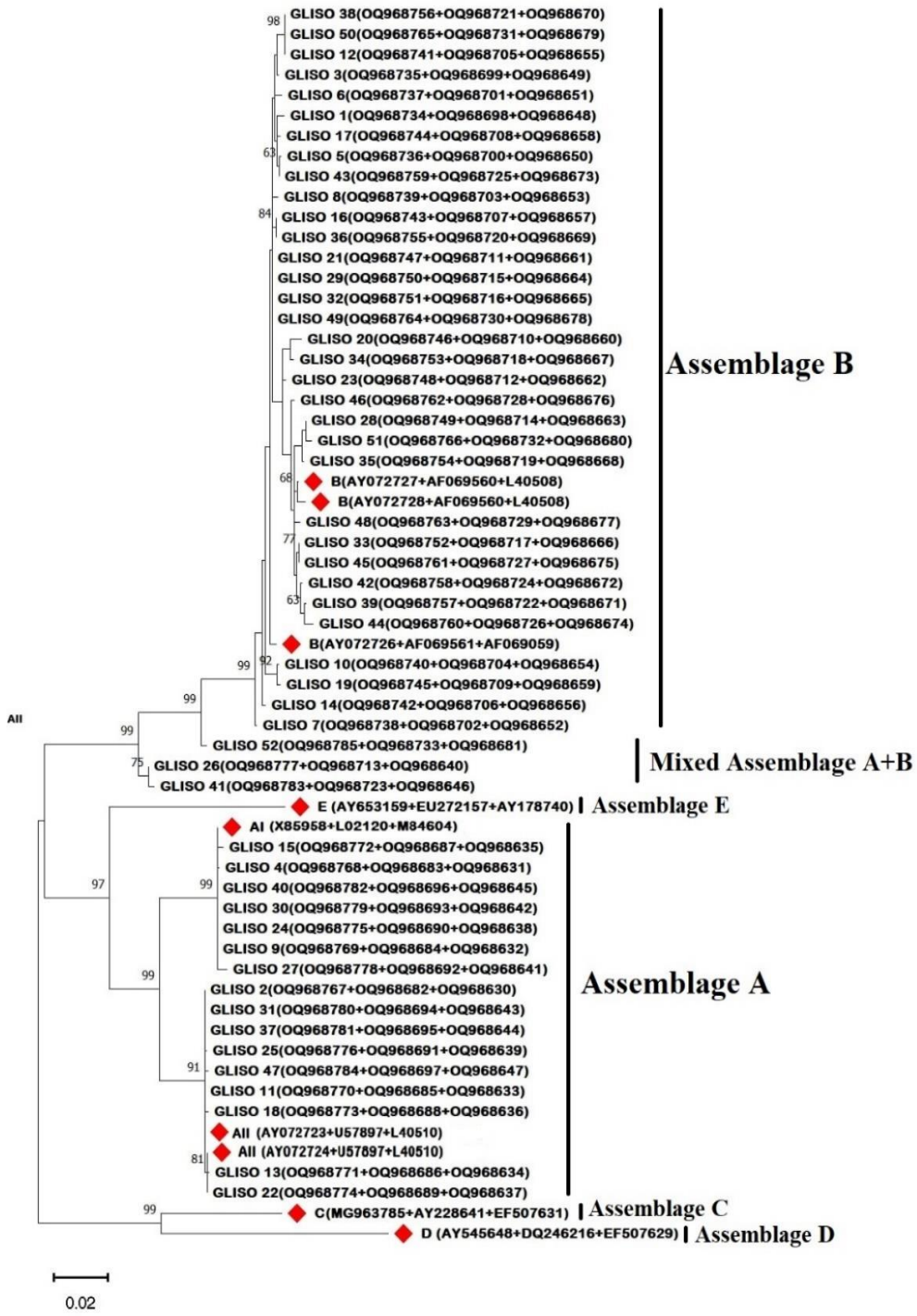


Fig. 4.6. Concatenated phylogenetic tree of study isolates based on nucleotide sequences of *bg, tpi* and *gdh* gene loci

4.3.4. Population structure

We obtained 16 individual sequences (1350bp) belonging to assemblage A. The alignment analysis revealed the presence of 10 distinct haplotypes and 22 polymorphic sites. The dataset exhibited high haplotype diversity (Hd) of 0.9±0.05 and low nucleotide diversity (π) level of 0.0036±0.0005. Among 33 sequences of assemblage B, 26 distinct haplotypes were obtained having a total of 45 polymorphic sites. High Hd value (0.97±0.01) accompanied with lower level of π (0.009±0.0005) were also obtained in this case (Table 4.3.). The presence of low nucleotide diversity alongside high haplotype diversity indicates the coexistence of numerous closely related haplotypes within the population. This observation suggests the possibility of a recent population expansion. However, it is important to note that, in case of assemblage A, lack of sufficient samples hampered the Fu's FS statistic to provide strong support for this finding. On the contrary, the Tajima's D statistics for assemblage B showed a positive value. This positive value could suggest the presence of either a population bottleneck or a balancing selection in play. The existence of a large number of diverse genotypes within this group, combined with the positive Tajima's D value, may support the hypothesis of balancing selection. However, since these values were not statistically significant, further confirmation of these results would require a larger sample size. To address the distribution and relationship of *Giardia* genotypes in different regions around Kolkata, 40 concatenated sequences were analyzed. The two assemblages displayed clear separation, forming distinct clusters with a substantial amount of polymorphism observed across the three partial fragments of housekeeping genes. This compellingly suggests that these two assemblages exhibit substantial differences from each other and can be appropriately classified as separate species. The presence of sub-clusters within both assemblage groups in the network provides evidence for the existence of subassemblages. Within the A assemblage, two distinct clusters, AI and AII,

showcased a notable stellate pattern. No such pattern was obtained for the haplogroups of assemblage B. This pattern strongly suggests an ongoing event of population expansion, as further supported by the neutrality test results. Due to the limited geographical boundary, the expected genotype clustering based on location was not observed. The 2 most abundant genotypes found, genotype 1A and 3A, both in similar frequency (N=4), were distributed in South 24 PGS, south Kolkata and central Kolkata, respectively. The haplogroup in assemblage B were named as haplogroup 1 and haplogroup 2. Among all the genotypes of this assemblage, genotype 10B was the most abundant (N=4) occurring in 2 out of the 8 study areas, specifically South 24 PGS and central Kolkata. The next most-abundant haplotype was genotype 6B (N= 3), which was found in 2 neighboring sites in east and southeast Kolkata. Genotype 12B (N= 2) and genotype 16B were exclusive to the relatively distant site of North 24 PGS. The haplotype network depicted in Fig. 4.7 shows numerous missing mutational steps, indicating the need for additional sampling in certain clusters, particularly in haplogroup 1 of assemblage B. Overall, the distribution of all genotypes in assemblage B also showed little geographic structure, as individuals from all sites were found to be mixed together.

Table 4.3: Summary table of Haplotype diversity of *Giardia* isolates (N= Number of Samples, S= Number of Sequences, H= Haplotypes, Hd = Haplotype diversity, Π = Nucleotide diversity, K = average FS= Fu's Fs, D= Tajima's D, S.D. = Standard Deviation, *P* = P value)

Population	**N**	**S**	**H**	**Hd (S.D.)**	**Π (S.D.)**	**K**	**FS (*P*)**	**D (*P*)**
Assemblage A	16	16	10	0.9 (0.05)	0.003 (0.0005)	4.967	-1.82 (0.08)	- 1.01 (>0.1)
Assemblage B	33	33	26	0.98 (0.01)	0.009 (0.0005)	12.212	-8.382 (<0.001*)	0.37 (>0.1)

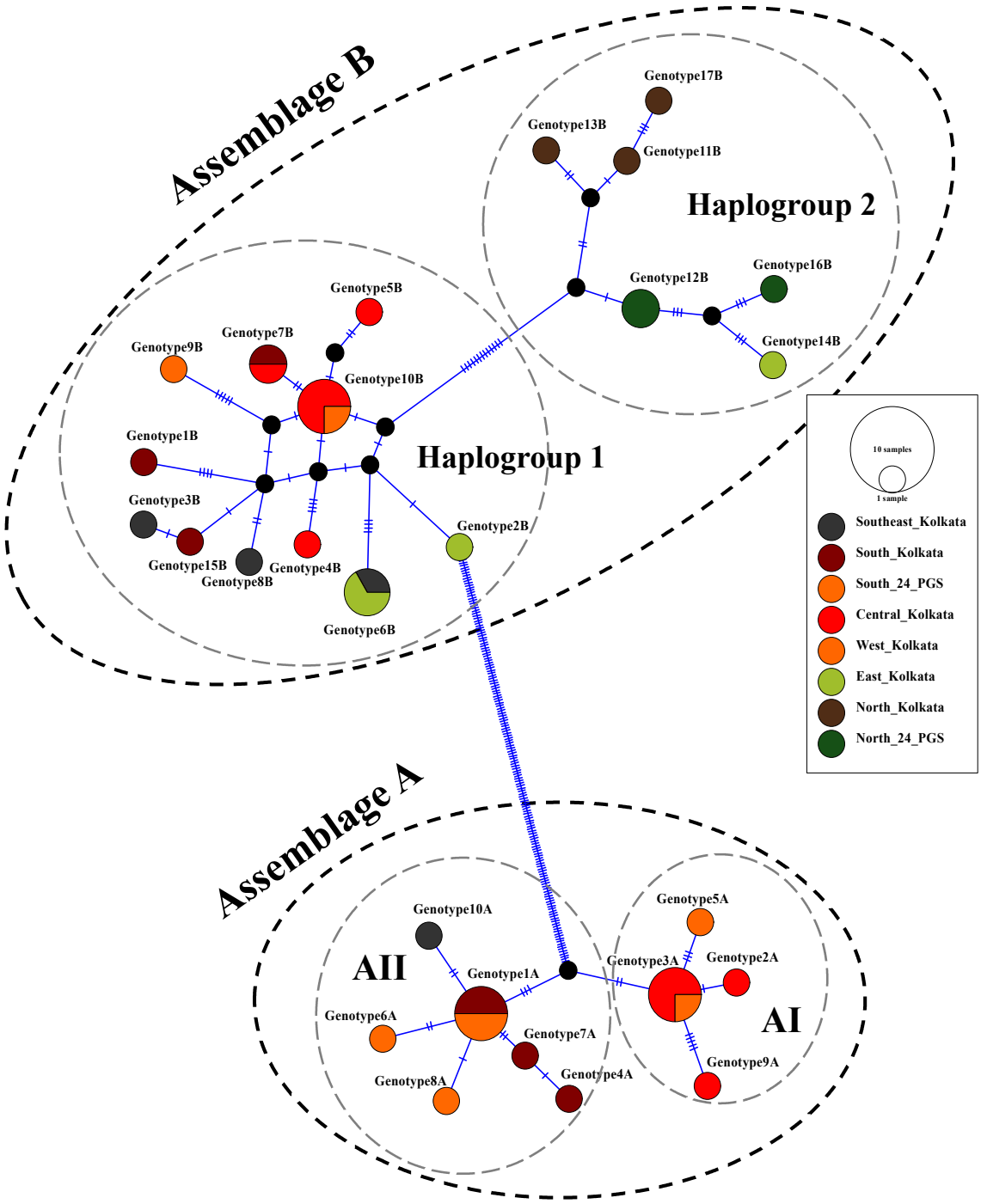


Fig. 4.7. A median-joining haplotype network was created with all the genotypes found in southeastern part of West Bengal. Haplotypes are depicted by circles of varying sizes based on the number of isolates. Geographic distribution is represented by different colours. Circles in black represent missing haplotypes

4.4. Discussion

This study aimed to investigate the genetic diversity of *G. lamblia isolates* among diarrheal patients in the southeastern region of West Bengal. According to this hospital-based survey, *G. lamblia* continues to be a prevalent pathogen commonly being found in diarrheal samples. Throughout this four-year study, we observed a steady rate of infection with a marginal rise in frequency during the monsoon season. For detection, microscopic observation of slides was carried out meticulously, followed by PCR. PCR is highly recommended (Emisiko et al., 2020), particularly in cases where samples initially show a low cyst load. In the study, PCR has demonstrated enhanced sensitivity allowing us to successfully detect and characterize *Giardia* genomic DNA in samples that were previously deemed negative under microscopic examination. Therefore, to achieve accurate positive results, it is recommended to utilize both microscopic examination and PCR simultaneously (Sari et al., 2019).

The present study reported a significant reduction in the prevalence of giardiasis or giardia-induced diarrhea in this region, showing a decrease from 13% to 6.3% compared to the previous decade (Mukherjee et al., 2009). This observed reduction in prevalence could be attributed in part to the improvements in water supply and sanitation implemented by the government of India, which may have contributed to partial eradication of giardiasis. However, despite the ongoing effort, the current prevalence rate of giardiasis among the children below 12 years of age remains alarmingly high. Individuals from economically disadvantaged (both rural areas and suburbs of Kolkata) regions had the highest infection rates. In low- and middle-income countries, giardiasis has been associated with impaired growth and poor cognitive function in malnourished children under the age of 5 (Shaima et al., 2021). A study in Mexico has correlated giardiasis with vitamin A deficiency in schoolchildren (Quihui-Cota et al., 2008). These reports point to the detrimental

effects of chronic *Giardia* infection on physical and mental development of the children, which ultimately impacts the overall human resource and economy of a country. In this scenario, more effective public health measures are needed to be taken, to address the ongoing concerns of *Giardia* infection in children in the region. This may include continuation of the awareness programs among parents and caregivers especially in low-resource settings about hygiene and precautions against parasitic infections.

The high occurrence of co-infection cases, found in the study setting, indicates that *G. lamblia* is adept at parasitizing the host gut while remaining asymptomatic. Lower prevalence of sole *Giardia* infections and the higher occurrence of co-infection cases with other enteric pathogens were found in this area. Several epidemiological studies undertaken in endemic regions around the world have reported a lack of positive correlation between *Giardia* infections and diarrhea. These findings highlight the fact that asymptomatic giardiasis is the norm rather than the exception in particular environments (Bartelt & Platts-Mills, 2016; Koster et al., 2021). These evidences shed light on the rationale behind the systematic exclusion of giardiasis from global burden estimates of diarrheal diseases (Global Burden of Disease Study 2013 Collaborators, 2015). Our data also supports the notion that only a minority of *Giardia infections* can lead to symptomatic disease and consequently highlights the importance of conducting community-based study in this area involving asymptomatic individuals to obtain a more accurate understanding of the giardiasis prevalence.

The genotyping results revealed the presence of both human infecting assemblages i.e., assemblage A and assemblage B in the study region. Assemblage B was found to be twice as common as assemblage A. These findings are consistent with a previous two-year study conducted in 2009-11 in the same region, which also reported a higher frequency of assemblage B compared to assemblage A in humans (Mukherjee et al., 2013). The level of diversity between these two

assemblages was found to be notably high. Increasing evidence from molecular studies supports the idea that these assemblages are indeed separate species (Thompson & Monis, 2012). Studies suggest that in humans, individuals are commonly affected by parasites belonging to either assemblage A or assemblage B (assigned a new species nomenclature as *G. enterica*), with the latter typically being more prevalent among human populations.

The global distribution of assemblage A and B exhibits varying prevalence rates across the world. Our study finding aligns with reports from various countries, such as Rwanda, Ethiopia, Zambia, the Brazilian Amazon, Spain, England, China and Qatar (Ignatius et al., 2012; ; Minetti et al., 2015; Wegayehu et al., 2016; Tembo et al., 2020; Köster et al., 2021; Wang et al., 2019; Yu et al., 2019; Chourabi et al., 2021), which have also documented a high prevalence of assemblage B. In contrast, Southwest Ethiopia (Gelanew et al., 2007), west Iran (Bahrami et al., 2017; Kashinahanji etal., 2019; Mahmoudi et al., 2020), southern Iran (Sarkari et al., 2012), Turkey **(**Aydin et al., 2004), Egypt (Hussein et al., 2016; Ahmad et al., 2020), Syria (Skhal et al., 2017), some parts of Brazil (Souza ei al., 2007**;** Figueiredo et al., 2020), and North America **(**Sulaiman et al., 2004) have exhibited a higher prevalence of assemblage A. Reports from Cuba (Pelayo et al., 2008), United Arab Emirates (El Bakri et al., 2014) and Southwestern Iran (Rayani et al., 2014; Rafiei et al., 2020**;**) revealed an equal distribution of assemblage A and B, which lines up with the findings of another study conducted in Pondicherry, India **(**Paintlia et al., 1998) . The high endemicity of assemblage B infection in the population of West Bengal indicates the successful parasitic adaptation of assemblage B in the region.

The clinical presentation of giardiasis exhibits considerable variability, with some individuals experiencing symptomatic manifestations such as acute or chronic diarrhea, nausea, flatulence, stomach cramps and weight loss while others remain asymptomatic (Muhsen & Levine, 2012). At

present it is difficult to establish a definitive correlation between the assemblages and clinical symptoms of giardiasis based on the limited studies. The distribution of *G. lamblia* genotypes appeared to differ between sole and mixed infection cases in this study. Furthermore, our findings indicate a probable association between assemblage A infection, and a higher odds ratio (OR) for giardiasis. Conversely, assemblage B infection was statistically linked to coinfection cases, which were observed at a significant rate (60%) within this population. In a study conducted in the neighboring country Bangladesh, a similar trend was observed, in finding assemblage A in sole diarrhea cases and assemblage B in cases having other coinfections (Haque et al., 2005). Multiple studies have suggested that Assemblage A infection is linked to more severe clinical symptoms in countries such as Australia, Great Britain, Syria, Egypt, Turkey and Iran (Read et al., 2002; Aydin et al., 2004; Breathnach et al., 2010; Alam et al., 2011; Sarkari et al., 2012; Pestechian et al., 2014; El Basha et al., 2016; Skhal et al., 2017). Our study also identified symptomatic giardiasis cases associated with assemblage A. This finding emphasizes the significance of diagnosing Assemblage A infection in individuals with diarrhea for effective infection control measures. However, conflicting results suggesting the opposite have been found in other studies from Netherlands, Ethiopia, Cuba, Malaysia, Saudi Arabia, UAE, Spain (Homan & Mank, 2001; Gelanew et al., 2007; Pelayo et al., 2008; Mohammed-Mahdy et al., 2009; Al-Mohammed 2011; El Bakri et al., 2014; Hussein et al., 2017; Wang et al., 2019). No association with either assemblage was found in studies conducted in Thailand, Portugal, Iran, Brazil and Nicaragua (Almeida et al., 2006; Kohli et al., 2008; Lebbad et al., 2008; Tungtrongchitr et al., 2010; Rafiei et al., 2020).

In a study in Egypt, giardiasis in children was shown to be statistically associated with assemblage B infection whereas, adults showed a similar frequency of harboring both assemblages (El Basha

et al., 2016). However, in this study, no significant correlation was found between age groups and the distribution of assemblages.

Consistent with previous reports, this study also revealed a significantly higher level of genetic diversity in assemblage B when compared to assemblage A (Sprong et al., 2009; Chourabi et al., 2021; Koster et al., 2021). For assemblage A, all the isolates belonged to two specific MLGs AI-A1 and AII-A2. Considering the association of sub-assemblage AI with various host species and the relatively restricted occurrence of sub-assemblage AII to humans (Rayani et al., 2013), our findings suggest that the transmission of Assemblage A in this region could be of both zoonotic and anthroponotic origin.

On the other hand, assemblage B isolates exhibited a higher level of diversity, with 26 different MLGs detected. This study highlights the presence of a wide variety of *Giardia* genotypes particularly, assemblage B in the southeastern part of West Bengal having significant number of novel genotypes, previously unidentified. Due to the large number of new variants found, no significant associations could be drawn between genotypes and demographic data of the patients. The regions of central, eastern, and southern part of Kolkata exhibited the highest abundance of genetic variants in this study. As the study participants were limited to a specific geographic area, it is of utmost importance to expand the study area and obtain a more comprehensive understanding of how these genotypes are distributed.

Gaining an understanding of the spatial genetic structure of populations plays a critical role in drawing conclusions about adaptive geographic variations in species and their capacity to adapt to future environmental changes. In order to evaluate the genetic diversity within populations, we estimated haplotype diversity (Hd) and nucleotide diversity (π), which determines the degree of polymorphism in two randomly sampled DNA sequences. For both assemblages A and B, low

nucleotide diversity (<0.01) and high haplotype diversity (≥0.9) were observed, suggestive of population expansion. The high levels of gene diversity observed in assemblage B compared to assemblage A suggests that the B population possesses a strong average fitness to withstand the effects of a changing environment. Such variations contributing to the overall fitness and adaptability of the population, provides potential advantageous traits that can enhance survival and reproduction in diverse environments. This may explain the better parasitic adaptation of B assemblage in comparison to assemblage A and higher endemicity as found in this study. The purpose of the neutrality tests is to differentiate between sequences that are evolving in a neutral manner under mutation-drift equilibrium and sequences that are subject to non-neutral processes viz., directional or balancing selection, as well as demographic expansion or contraction (De Jong et al., 2011). For both populations, a negative value of Fu's Fs indicates an excess of diverse haplotypes than be expected under neutrality, typically associated with a recent expansion in the population. The Tajima's D test for assemblage A population yielded a statistically significant negative result, indicating an excess of rare nucleotide variants than expected number under a neutral model of evolution. This suggests recent historical demographic expansion in the population. The presence of a stellate pattern within the haplotype network also supports this claim (Sardar et al., 2023). In contrast, the assemblage B population exhibited a positive value of Tajima's D, although it was not statistically significant. This positive value suggests that relatively low levels of both low and high frequency polymorphisms are present within the population, indicative of balancing selection pressure in operation. This result is in accordance with our observations, as the presence of balancing selection actively preserves multiple alleles for a longer period than expected by random chance. This contributes to an increased average fitness of the population.

Multi-locus PCR is widely recognized as the primary method for effective genotyping of *G. lamblia*, as opposed to single locus genotyping, which fails to identify cases of mixed assemblages. MLST analyses focusing on the housekeeping genes such as, *bg, gdh*, and *tpi* loci have led to the rapid identification of multiple subtypes (Xiao et al., 2017). Assemblage A, in particular, encompasses six distinct subtypes, namely A1- A6. These subtypes are classified under the three sub-assemblages: AI comprises A1 and A5; AII includes A2, A3, and A4; and AIII consists of A6. The differentiation between these subtypes often relies on the detection of a single point mutation, signifying minor genetic variations. On the contrary, interpretation of genotyping results for assemblage B using this method is complex due to the extensive genetic variability and high allelic sequence heterogeneity (ASH) present in this assemblage (Zajaczkowski et al., 2021). High genetic diversity found in our study also provides evidence to support this statement. Besides, distinguishing between monotypic infections involving a strain with high ASH and infections with multiple strains with low ASH becomes a challenging task using this approach. Therefore, distinct strategies are necessary for achieving the same level of resolution in molecular typing of assemblage A and B.

The phenomenon of "assemblage swapping" has been observed in the study which was linked to a potential outcome of mixed-assemblage infections. We encountered cases of mixed assemblages (A+B= 5.7%), which posed challenges in accurately assigning specific genotypes to these isolates. Mixing within assemblage can occur when genetic exchanges take place between different assemblages within a single cyst, resulting in the formation of hybrid recombinants. Since conclusive evidence of sexual recombination between different assemblages has not been established, it is more probable that these cases represent instances of mixed infections. Consequently, in order to ensure accuracy, we excluded these cases while assigning genotypes.

Single cyst analysis can potentially overcome the challenge associated with detection of multiple infections, although it is not practically feasible in all settings (Woschke et al., 2021).

Initially, *Giardia* was thought to reproduce exclusively through asexual means (Zajaczkowski et al, 2021). However, studies on whole-genome sequencing have identified genes associated with meiosis, indicating the potential for nuclear fusion (karyogamy) and subsequent somatic recombination during the cyst stage (Ramesh et al., 2005; Poxleitner et al., 2008). Furthermore, both epidemiological studies (Teodorovic et al., 2007; Lasek-Nesselquist et al., 2009) and population genetic data (Cooper et al., 2007; Kosuwin et al., 2010) provide strong evidence supporting recombination within and between varying *G. lamblia* isolates, although direct observation confirming it is yet to be attained. It is likely that sexual reproduction is an occasional event and may only occur under specific conditions. Besides, studies have found allelic sequence heterogeneity (ASH) levels in assemblage A is as low as 0.01-0.03% and 0.4-0.5% in assemblage B. (Cooper et al., 2007; Morrison et al., 2007; Teodorovic et al., 2007; Adam et al., 2013; Xu et al., 2020). This observation supports with the idea that sexually reproducing organisms would maintain low level of allelic heterozygosity due to the requirement of chromosome pairing during meiosis. Conversely, asexual organisms with a ploidy of two or more would exhibit highly divergent allelic sequences due to the independent evolution of the nuclei (Zajaczkowski et al, 2021). These findings suggest that there remains a possibility of genetic exchange in this parasite, which may have contributed to its adaptability to diverse environments encountered and could potentially alter the parasite's characteristics through modifying the genes associated with drug tolerance.

4.5. Conclusion

In this study, we determined the prevalence of *G. lamblia in* diarrheal patients in a limited geographical boundary of West Bengal. The results highlight the vulnerability of children below 12 years of age to this parasitic infection emphasizing the need for continued awareness on maintaining hygiene. MLG analysis of the isolates, with recommended genetic markers, revealed that the frequency of assemblage B was twice as high as assemblage A in this region. The study also revealed a potential correlation between Assemblage A and symptomatic giardiasis infections. The extensive genetic variations observed within assemblage B made it challenging to determine specific MLGs and accurately assign the isolates to established sub-assemblages. Furthermore, population structure analysis revealed that assemblage A population had experienced recent population growth whereas assemblage B population is under balancing selection having greater adaptability to the diverse environment that these parasites encounter. For a better understanding of genetic diversity and transmission dynamics within *G. lamblia* assemblages, conducting further community-based studies involving a larger sample size and including animals and environmental samples in state of West Bengal is recommended. Moreover, our study emphasizes the importance of employing advanced molecular biological tools to accurately identify *Giardia* genotypes and subtypes, as well as their distribution.

Chapter 5

PREVALENCE AND GENOTYPING OF G*iardia lamblia* LOCAL ISOLATES FOUND IN ASYMPTOMATIC POPULATION

5.1. Introduction

Giardia lamblia (*G. lamblia*) significantly contributes to the global burden of diarrheal disease, resulting in nearly 280 million cases of Giardiasis annually. This leads to the loss of approximately 171,100 daily-adjusted life years. Giardiasis stands out as the most frequently reported intestinal protozoan infection worldwide (Koster et al., 2021). Clinical manifestations vary from severe symptoms such as diarrhea, flatulence, abdominal pain, vomiting, anorexia, malabsorption and weight loss to an asymptomatic carrier state. Long-term consequences, including postinfectious irritable bowel disease and chronic fatigue, have been documented (Tijani et al., 2023). Chronic infection is linked to malabsorption, potentially leading to weight loss and wasting in children (Squire and Ryan, 2017). As per the definition, asymptomatic infections encompass instances that result in malabsorption/malnutrition or may only represent pure colonization without pathology (Kraft et al., 2017). *G. lamblia* is also associated with cognitive impairment in school-age children (Tembo et al., 2020). The control of the infection or its progression to active disease appears to be influenced significantly by the immune status and nutritional level of the host. The genotype of the parasite may contribute to the overall health-disease balance in the host, although definitive association is still to be established (Koster et al., 2021). Reports from developing countries showed that giardiasis is linked to inadequate sanitary conditions, poor water quality, and overcrowding. Conversely, in developed countries, cases are typically connected to international travel and immigration. Analysis of fecal samples indicates a higher prevalence, approximately 20-30%, among symptomatic cases in developing countries, in contrast to 2-5% in the developed world. (Nikolić et al., 2011). Available estimates of the proportion of asymptomatic infections, with the potential for continuous transmission of disease is limited and vary considerably from 5 to 15% (Hill, 2001) to 76% (Lopez et al., 1980) in certain reports. Large epidemiological case-

control studies in high-prevalence settings have shown that *G. lamblia* infection is significantly more common in asymptomatic controls than in cases with diarrhea (Koster et al., 2021). Several other studies have found that *Giardia* infection is negatively associated with hospitalized diarrhea (Bilenko et al., 2004; Kotloff et al., 2013; Muhsen et al., 2014; Breurec et al., 2016). Expert consensus emphasizes the importance of considering treatment for asymptomatic cases in specific situations, including instances involving food handlers, day care nurseries, and recurrent infections within a household (Waldram et al., 2017). Prior investigation into the recent epidemiological situation of *G. lamblia* among diarrheal patients in the southeastern part of West Bengal revealed the substantial public health concern posed by this protozoan parasite, exhibiting a prevalence rate of 6.78%. Infection in asymptomatic individuals has been inadequately explored in this region, partly due to a lack of general awareness, the absence of noticeable symptoms, and hence a perception that these cases are less urgent for treatment. Additionally, incomplete understanding of the progression and outcomes of the disease contributes to the neglect of asymptomatic cases. The persistence of infectivity for months without treatment raises the potential for household clusters or outbreaks. Currently, there is no evidence-based guidance on the optimal timing for treating asymptomatic infection to curb transmission, and asymptomatic carriage is typically untreated. This study seeks to gauge the prevalence of *Giardia* infection in households with index cases of giardiasis, identify characteristics of the infected community, and offer a more comprehensive understanding of transmission dynamics and risk factors. We again utilized the conventional multilocus genotyping method to genotype the *G. lamblia* isolates circulating in this limited boundary of Kolkata and nearby districts.

5.2. Materials and Methods

5.2.1. Sample Collection and Ethical Statement

For this study, we obtained non-diarrheal fecal samples collected between June, 2021 to September, 2023, during a community-based project aimed for surveillance of foodborne parasite infection (Ref No. NER/62/(3)/2018-ECD-I). The study was approved by the Institutional Ethics Committee of ICMR-National Institute of Cholera and Enteric Diseases. Individual consent was obtained during the survey before enrollment and the samples were coded anonymously to preserve participants confidentiality in accordance with study ethics. Inclusion criteria for the samples in this study were: 1) collected from individuals residing in the region for over a year, 2) from individuals that had not undergone treatment with antiparasitic drugs and/or anti-diarrheal preparations for at least one month prior to collection, 3) samples which exhibited Bristol stool type 3-5, 4) of the individuals whose comprehensive details were present. These samples were collected from different study sites, which included the slum population of Kolkata, the rural and urbanized areas of South 24 PGS and North 24 PGS (Fig. 5.1).

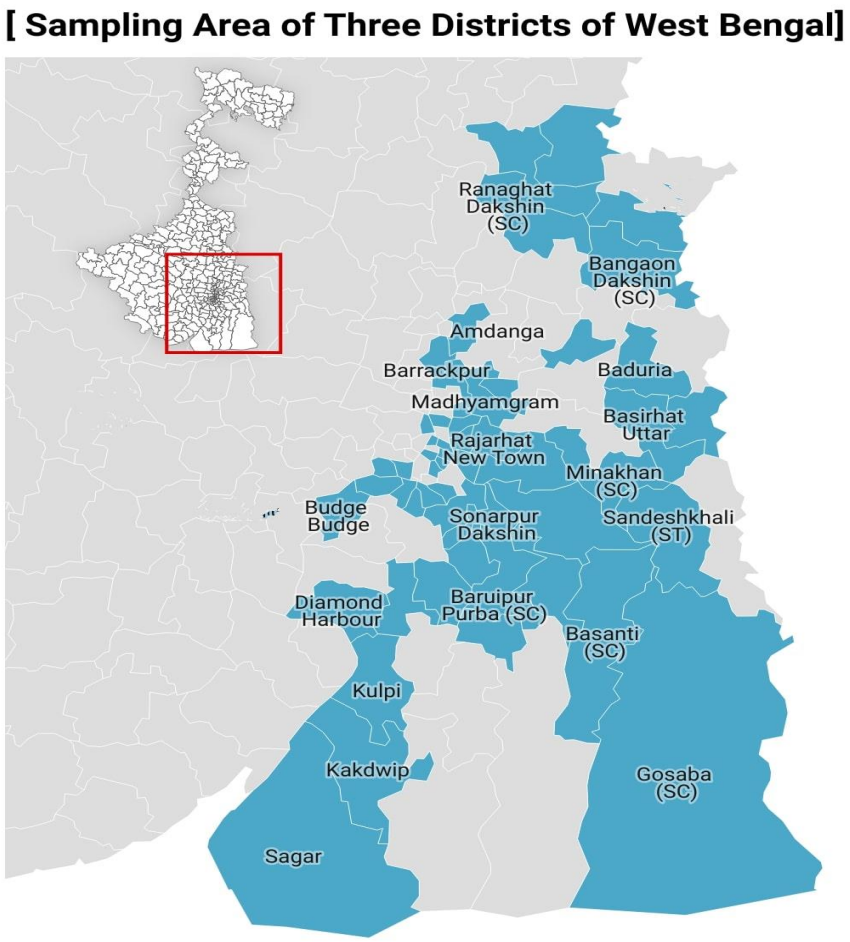


Fig 5.1: Map of West Bengal, showing sampling areas in Kolkata, South 24 Parganas and North 24 Parganas

5.2.2. Data Collection

Comprehensive details regarding individuals, including age, gender, clinical symptoms, household location, source of drinking water, hygiene practices, animal contact etc., were extracted from the study records. Stool specimens were visually examined to categorize into consistency types 3 to 5. During sample collection each participant was allocated a unique identifier, facilitating the correlation between their stool samples and respective epidemiological questionnaires.

5.2.3. Laboratory analyses

5.2.3.1. Microscopy

Microscopic examination was performed on stool samples to detect *G. lamblia* and other intestinal parasites, following the formol-ethyl acetate stool concentration method. Concentrated fecal samples were examined by preparing wet mount slides with iodine. Each sample underwent a meticulous analysis under a high dry (40×) objective, utilizing a phase-contrast microscope (Olympus, Tokyo, Japan).

5.2.3.2. Stool DNA Isolation

Before concentration, the raw stool samples were aliquoted into 2ml microcentrifuge tubes for DNA isolation. Total DNA extraction from the fresh samples was performed using the QIAamp DNA Stool Mini Kit (Qiagen, USA), following the manufacturer's protocol. The eluted DNA was then preserved at -20°C until needed.

5.2.3.3. PCR Confirmation and Genotyping of *G. lamblia*

Confirmation of *G. lamblia* infection was established through a conventional PCR method that targets a 218-bp region coding for the beta giardin (*bg*) gene. Samples that exhibited a positive result in PCR were subjected to a re-evaluation through sequence-based multi-locus genotyping. This involved the examination of genes responsible for encoding the glutamate dehydrogenase (*gdh*) and triosephosphate isomerase (*tpi*) proteins of *G. lamblia*. The PCR protocols and necessary primers were detailed in the previous chapter. PCR products were separated through 1.5% agarose gel electrophoresis and visualized following ethidium bromide staining.

5.2.3.3. DNA Sequencing

Purified PCR products were subjected to bidirectional Sanger-dideoxy sequencing using ABI BigDye Terminator v. 3.1 sequencing kit (Applied Biosystems, USA) on an ABI 3730 PRISM® DNA Analyzer (Applied Biosystems, USA). The conditions for sequencing PCR and PCR purification were specified in previous chapter.

5.2.4. Phylogenetic analysis

For the identification of *G. lamblia* genotypes, the nucleotide sequences obtained for each gene were aligned with reference sequences (GL50803_93938, L02120, KR051228, GU564274, DHA2_93938, U57897, KJ888993, DQ650648, GL50803_21942, KP687782, DHA2_21942, KJ741313, L40510 and EU637582 for assemblage A; AF069561, AF069560, AY368165, AY368163, AY228628, GSB_93938, L02116, AF069059, JQ700429, GSB_21942, EU834843, EU594666, DQ090541, AY826192, L40508 for assemblage B) from NCBI GenBank. This alignment was performed using ClustalW in MEGA11. For the phylogenetic analysis of the sequences obtained, a concatenation of *gdh* and *tpi* loci was utilized. Evolutionary relationships were established using the maximum likelihood (ML) method, employing the Tamura Nei parameter model in MEGA11 to calculate evolutionary distances. The resulting trees were assessed for robustness through the bootstrap method with 100 pseudo-replicates, and only values exceeding 50% were reported.

5.2.5. Genetic Diversity of *G. lamblia* isolates

DnaSP v5.0 was employed to compute various genetic diversity measures from the aligned sequences, encompassing the haplotype diversity (Hd), number of haplotypes (h), variable sites (S) and nucleotide diversity (π). To comprehend and compare the relationships among *G. lamblia*

genotypes identified in diarrheal cases (from a previous study) and non-diarrheal cases, a median-joining haplotype network was constructed using PopART v1.7. Isolates were color-coded based on distinct outcomes, facilitating the inference of relationships with the sequenced data. Additionally, two neutrality tests (Fu's FS and Tajima's D) were performed using DnaSP v5.0 to evaluate the population dynamics within the *Giardia* assemblages.

5.2.6. Statistical Analysis

We explored factors (public health features) linked to a positive *G. lamblia* result. Data analysis was carried out using GraphPad Prism v.9.0.0 (CA, USA) software. The Fisher's exact test was utilized to compare the sociodemographic parameters of the individuals with their *G. lamblia* infection status. The tests were two-sided, and a p-value<0.05 was deemed significant.

5.3. Results

5.3.1. Sociodemographic Characteristics of study population

In this study, a total of 1,479 fecal samples met the inclusion criteria during the sampling period, with 692 samples collected in the first year and 787 samples in the second year. The gender ratio of the individuals was 1.42 (male/female), and their ages ranged from 2 years and 9 months to 83 years (median age: 30 years). The most represented age group was >20-40 years, comprising 35% of the population (n/N=518/1,479 individuals). Additionally, 55.7% of the participants resided in rural areas, and 42.1% were from low and lower-middle socioeconomic strata. Regarding education, 14.7% had no formal education, 15.6% had primary education, 23.4% had intermediate education, and 46.1% had higher education. 41.3% of the individuals had contact with animals. Furthermore, 21.4% had the habit of washing or bathing in open water reservoirs. The distribution of stool types among the fecal samples was as follows: 20% were classified as stool type 3 (ST3),

47.6% as stool type 4 (ST4), and 32.4% as stool type 5 (ST5). All samples were screened for the detection of *G. lamblia*.

5.3.2. Prevalence and features of *G. lamblia* infection

G. lamblia cysts were detected in 8.2% of the fecal samples (121/1,479; 95% CI: 6.8–9.6%) using conventional microscopy (Fig 5.2.). PCR screening (Fig 5.3.) identified *G. lamblia* in a total of 153 positive samples, including all microscopy-positive cases, yielding an overall prevalence of 10.3% (153/1,479; 95% CI: 8.8–12.0%). The study observed the highest incidence of *G. lamblia* infections during the monsoon season of each year, with 14.35% in 2021, 15.55% in 2022, and 12.71% in 2023. In contrast, the dry or winter season exhibited a substantial decrease in the percentage of positive cases, with 3.14% in 2022 and 4.02% in 2023 (Fig 5.4.).

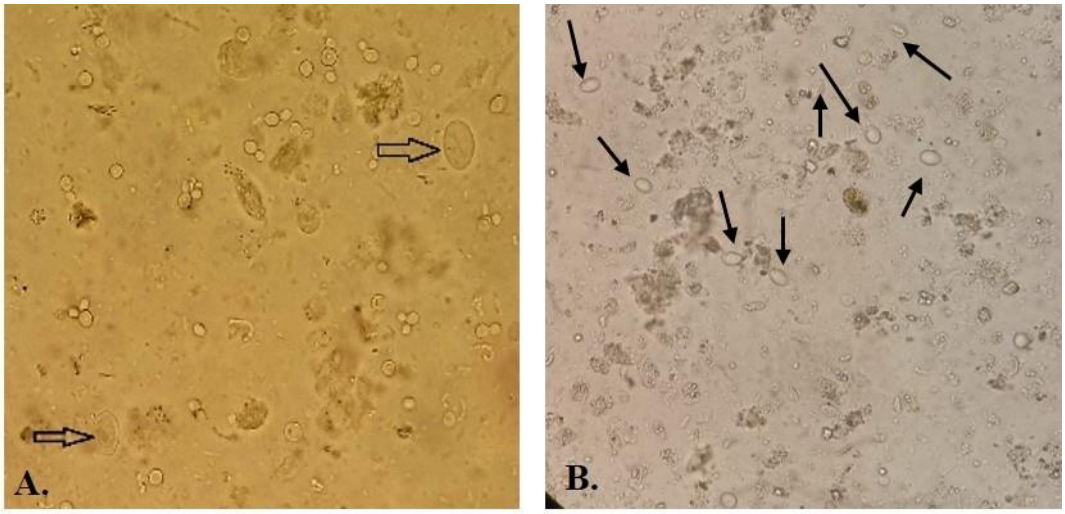


Fig 5.2. Cyst load of *Giardia lamblia* in fecal samples of asymptomatic individuals, wet mount [Iodine] (40x). A. Sample with low cyst load (+); B. Sample with moderate cyst load (++).

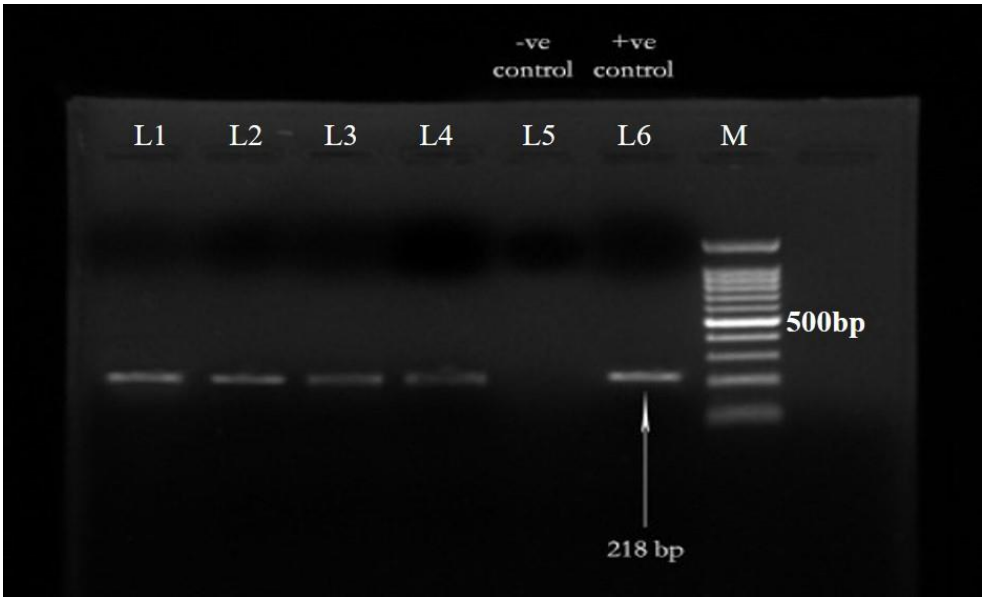


Fig 5.3. PCR product on agarose gel stained with ethidium bromide for *Giardia lamblia* detection from clinical samples. L 1-4: *Giardia* positive samples (218bp); L5: Negative control; L 6: Positive control; M: 100bp marker

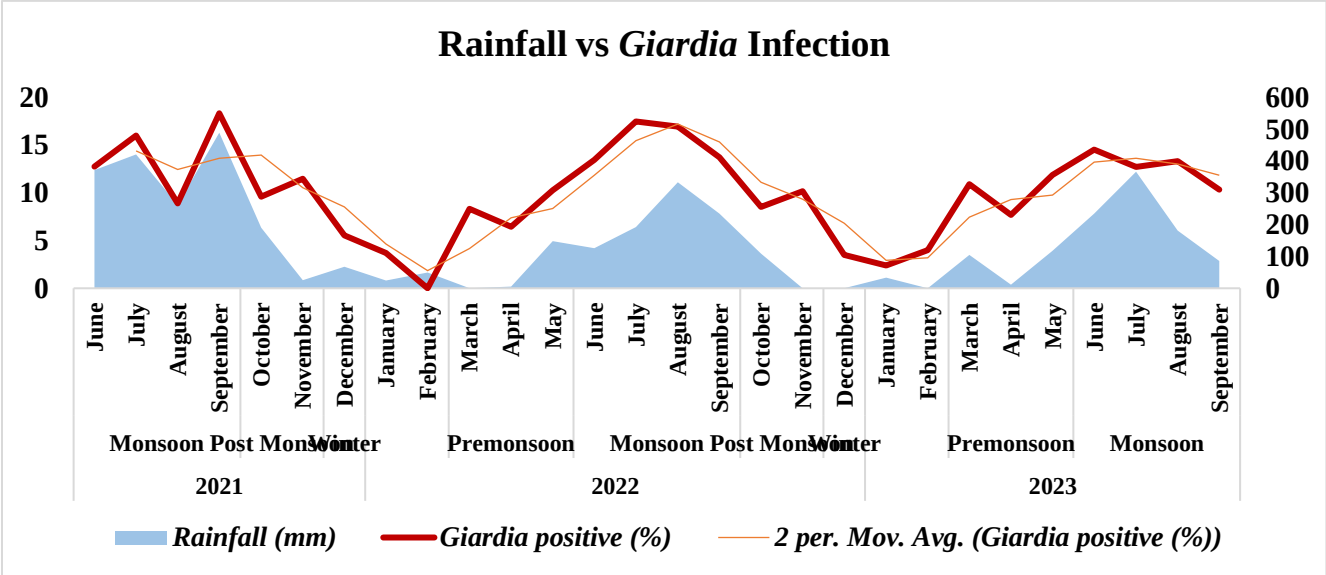


Fig 5.4. Seasonal distribution of *G. lamblia* infection in the study region

G. lamblia infections were most prevalent in the age group >2–10 years (reference category; 15%), followed by >10–20 years (12.7%) and >60 years (11.3%). In contrast, the age groups >20–40 and >40–60 years had significantly lower infection rates, at 8.3% (OR=0.52; 95% CI: 0.32–0.86, P=0.01) and 8.13% (OR=0.5; 95% CI: 0.28–0.31, P=0.02), respectively.

Moreover, individuals from economically disadvantaged backgrounds and those with no more than primary level education exhibited the highest infection rates. The highest rate of positive cases (20.6%; OR=14.7; 95% CI- 5.9-36.7; P<0.001) was observed in fecal specimens exhibiting a type 5 consistency (ST5). The distribution of *Giardia* infection according to various demographic and environmental variables (gender, age group, area of residence, literacy, socioeconomic status, animal contact, source of bathing/washing water, and stool consistency) is summarized in Table 5.1.

Table 5.1. Binary logistic regression models exploring risk factors for *G. lamblia* infection (Value analyzed by Fisher's exact test is considered statically significant when $p < 0.05$)

Category	N	% Positive	OR	95% CI	P value
Gender					
Male	868	10.71	1.1	0.78-1.55	0.57
Female	611	9.810	-	-	-
Age groups					
>2-10	182	15	-	-	-
>10-20	324	12.65	0.83	0.49-1.4	0.490
>20-40	518	8.3	0.520	0.31-0.86	**0.01***
>40-60	295	8.13	0.508	0.28-0.91	**0.02***
>60	160	11.25	0.728	0.38-1.37	0.320
Residential Area					
Rural	824	14.92	3.65	2.42-5.53	<**0.001***
Urban	655	4.58	-	-	-
Literacy					
No education	217	19.81	-	-	-
Primary	232	16.81	0.81	0.5-1.32	0.46

Intermediate	347	12.68	0.58	0.37-0.92	**0.03***
Higher	683	3.95	0.16	0.1-0.27	**<0.001***
Socioeconomic Strata					
Lower	363	18.45	12.947	5.14-32.58	**<0.001***
Lower Middle	260	16.92	11.652	4.5429.88	**<0.001***
Middle	565	6.54	4.008	1.55-10.31	**0.004***
Upper	291	2.06	-	-	-
Animal Contact					
Yes	612	10.13	0.960	0.68-1.352	0.82
No	867	10.49	-	-	-
Bathing/Washing water source					
Open Water Source	317	34.06	12.82	8.79-18.71	**<0.001***
Enclosed Water Source	1162	3.87	-	-	-
Stool Consistency					
ST3	296	1.68	-	-	-
ST4	704	6.96	4.54	1.79-11.5	**0.001***
ST5	479	20.66	14.77	5.93-36.77	**<0.001***

5.3.3. Genotyping and assemblage/sub-assemblage distribution

From a total of 153 molecular-positive samples, 46 positive specimens with moderate cyst loads were selected and evaluated for the amplification of 531bp and 432bp fragments of the *tpi* and *gdh* genes, respectively, using a Nested-PCR approach (Fig 5.5.). Among the 46 examined samples, PCR amplification was successful for the *tpi* gene in all 46 samples (100%), while amplification of the *gdh* gene was achieved in 41 samples (89%). Concomitant amplification of both genes was observed in 41 samples (89%). Phylogenetic analysis revealed the presence of two assemblages, A (n/N=16/46; 34.8%) and B (n/N=26/46; 56.5%), encompassing four sub-assemblages (AI, AII, BIII, and BIV) among the infected individuals. Mixed assemblage cases (A+B) were identified in

8.7% (n/N=4/46) of the samples. The assemblage/sub-assemblage characterization of all 46 examined samples was accomplished by genotyping at least one gene locus. Accordingly, 15.2% (n/N=7/46 samples) of the positive cases belonged to sub-assemblage AI, 19.6% (n/N=9/46 samples) were classified as sub-assemblage AII, 34.8% (n/N=16/46 samples) were identified as sub-assemblage BIII, 17.4% (n/N=8/46) of the positive samples were attributed to sub-assemblage BIV, and 4.34% (n/N=2/46 samples) were detected as sub-assemblage BIII/BIV (Table 5.2.). No host-specific assemblages of canine, feline, or livestock (C-F) origin were detected in the examined samples.

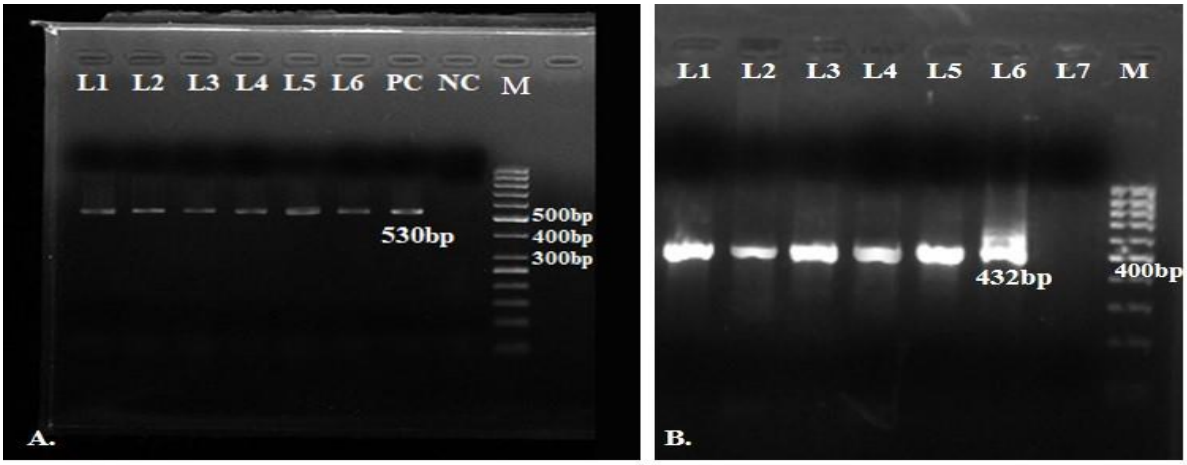


Fig 5.5. Amplification of *Giardia lamblia* triose phosphate isomerase (*tpi*) (A) and glutamate dehydrogenase (*gdh*) gene fragments (band obtained at 531 and 432bp, respectively). M: 50bp marker.

Among the 46 *tpi* sequences analyzed, 10 were assigned to the sub-assemblage AII_A2, and 7 were assigned to the sub-assemblage AI_A1. Within the 10 AII isolates, 8 exhibited 100% sequence similarity to the reference sequence for AII_A2 (DHA2_93938 and U57897), while one sample (ND 166) displayed single nucleotide polymorphisms (SNPs) at position 445 (G/A). (ND179) also had a single nucleotide polymorphism (SNP) at 445 (G/A) along with another SNP

at 52 (C/T). All isolates classified as sub-assemblage AI_A1 demonstrated 100% sequence identity to the reference sequence (GL50803_93938 and L02120).

Out of the 41 *gdh* sequences, 11 were assigned to the sub-assemblage AII_A2, and 6 were assigned to the sub-assemblage AI_A1. Among the 11 AII isolates, 9 exhibited 100% sequence similarity to the reference sequence for AII_A2 (DHA2_21942 and L40510), while the remaining 2 samples (ND4 and ND89) displayed a single nucleotide polymorphism (SNP) at position 549 (G/A). All isolates classified as sub-assemblage AI_A1 demonstrated 100% sequence identity to the reference sequence (GL50803_21942).

Regardless of the molecular marker employed, the genetic diversity observed within the assemblage B sequences was considerably higher than that observed within the assemblage A sequences. Out of the 46 samples analyzed, 26 isolates belonged to the assemblage B. Multiple sequence alignment at the two loci revealed the presence of single nucleotide polymorphisms (SNPs) at multiple sites across the sequences when compared to the reference sequences. The number of varying sites identified was 11 at the *gdh* locus and 14 at the *tpi* locus.

When analyzed at the *tpi* locus, it was found that 29 out of 46 isolates belonged to the assemblage B. Alignment analyses with reference sequences revealed that 19 isolates were of the BIII subassemblage. Among them, 12 isolates exhibited 1-2 single nucleotide variations at positions such as 504 C/T (ND4, ND35, ND83); 45 T/C, 162 G/A (ND9); 162 G/A (ND22); 162 G/A, 354 G/A (ND72, ND123). Seven isolates demonstrated 100% sequence identity with the reference sequence AF069561. ND145 and ND29 both harbored a single nucleotide polymorphism (SNP) at the hotspot site or subassemblage-defining position, i.e., 39 G/A, with ND29 having an additional SNP at position 362 G/A, and they were classified as BIII-like variants. Sequences of 3

isolates (n/N=3/29, 10.3%) presented overlapping nucleotide peaks at one hotspot position 165 C/Y (ND 133, ND 156). Among the three, isolate ND 250 had another SNP outside the hotspot site at 216 C/T. Since only one double peak was obtained in the hotspot site, it did not interfere with the characterization of the isolates as sub-assemblage BIII and finally assigned them as BIII/BIII-like. 8 isolates were identified as belonging to the subassemblage BIV. 2 isolates (ND52, ND182) exhibited 100% sequence similarity with the reference sequence AF069560. The remaining isolates harbored single nucleotide polymorphisms (SNPs) at positions as follows: ND173 and ND211 exhibited SNPs at positions 210 A/G and 269 G/A. Remaining 4 isolates (ND27, ND94, ND114, ND196) presented overlapping nucleotide peaks at positions 111 G/R and 165 C/Y. As double peaks were found at only one hotspot position, these isolates were classified as BIV/BIV-like variants. 2 sequences (ND65, ND115) were identified as ambiguous BIII/BIV sequences, and they differed from reference sequences by 5-6 single nucleotide polymorphisms. Most SNPs detected in them corresponded to double peaks at hotspot single nucleotide positions, indicating heterozygosity.

Of the 41 amplified and characterized *gdh* gene sequences, 27 belonged to assemblage B. When compared to the reference sequences AF069059.1 and JQ700429.1, 5 isolates showed 100% identical sequence matches, indicating complete sequence similarity. However, 10 isolates exhibited between 1-2 single nucleotide polymorphisms (SNPs) at different positions. Isolate ND15 harbored one SNP at position 534 (A/G), while 4 isolates (ND65, ND133, ND156, ND250) had a SNP at position 406 (T/C). Nd72 and ND123 both harbored a SNP at position 519 (C/T). 2 isolates (ND 103 and ND 201) exhibited double peak at a hotspot position 309 (C/Y) and were assigned BIII-like subassemblage. Another isolate ND22 displayed overlapping nucleotide peaks at three positions 309 (C/Y), 429 (T/Y) and 540 (C/Y). Consequently, ND22 was classified as a

BIII/BIII-like variant of *G. lamblia*. Among the isolates analyzed, 8 were identified as belonging to the sub-assemblage BIV based on the *gdh* locus. Four isolates (ND27, ND52, ND114, ND166) exhibited 100% sequence similarity with the reference sequence L40508. The remaining isolates harbored single nucleotide polymorphisms (SNPs) at various positions. Specifically, ND94 and ND196 exhibited SNPs at position 597 (C/T), while ND211 had an SNP at position 406 (T/C). The isolate ND182 presented an overlapping nucleotide peak at the hotspot position 309 (C/Y) and was consequently classified as a BIV/BIV-like variant. Here, 1 sequence (ND115) was classified as an ambiguous BIII/BIV sequence, displaying double peaks at hotspot positions, suggesting the presence of mixed alleles or heterozygosity.

The analysis of multilocus genotypes (MLGs) in the 46 isolates revealed extensive genetic diversity, with numerous heterogeneous positions observed within each gene fragment. The study encountered four mixed assemblage isolates (ND4, ND41, ND83, ND166) and two mixed subassemblage isolates (ND4, ND83). To ensure accurate genotype assignment, these isolates of mixed assemblage were excluded from the analysis. Among the 16 successfully genotyped assemblage A samples at both genetic loci, a total of four MLGs were identified (Table 5.2). The MLGs belonging to the AII sub-assemblage were dominant, detected in 9 samples, while the remaining MLGs were all of the AI sub-assemblage, found in 7 samples. No mixed sub-assemblages were detected within assemblage A. Among the 30 successfully genotyped assemblage B samples at both genetic loci (Table 5.2), a total of 14 MLGs were detected. 9 multilocus genotypes (MLGs) belonging to the BIII sub-assemblage were predominant, being detected in 14 samples. The remaining 5 MLGs were attributed to BIV sub-assemblage, found in 7 samples. The dominant MLG was MLG-B8, observed in 3 samples. MLG-B4, MLG-B7, MLG-

B9, MLG-B10, and MLG-B11 were each detected in 2 samples. The remaining MLGs were each detected in only one sample (Table 5.2).

Table 5.2.: Multilocus genotyping result of all the *Giardia*-positive samples

Sl no	Isolates	*tpi*	*gdh*	MLG
1	ND4	BIII	AII_A2	BIII+AII
2	ND9	BIII	BIII	BIII
3	ND15	BIII	BIII	BIII
4	ND17	AII_A2	AII_A2	AII
5	ND22	BIII	BIII/BIII-like	BIII
6	ND25	AI_A1	-	AI
7	ND27	BIV/BIV-like	BIV	BIV
8	ND29	BIII-like	-	BIII
9	ND35	BIII	BIII	BIII
10	ND38	AII_A2	AII_A2	AII
11	ND41	BIII	AII_A2	BIII+AII
12	ND45	AI_A1	A1_A1	AI
13	ND48	AII_A2	AII_A2	AII
14	ND52	BIV	BIV	BIV
15	ND56	AII_A2	AII_A2	AII
16	ND61	AI_A1	A1_A1	AI
17	ND65	BIII/BIV	BIII	BIII/BIV
18	ND72	BIII	BIII	BIII
19	ND83	BIII	AI_A1	BIII+AI
20	ND88	BIII	BIII	BIII
21	ND89	AII_A2	AII_A2	AII
22	ND94	BIV/BIV	BIV	BIV
23	ND99	AII_A2	AII_A2	AII
24	ND103	BIII	BIII-like	BIII
25	ND109	AI_A1	-	AI
26	ND114	BIV/BIV-like	BIV	BIV
27	ND115	BIII/BIV	BIII/BIV	BIII/BIV
28	ND120	AII_A2	AII_A2	AII
29	ND123	BIII	BIII	BIII
30	ND133	BIII/BIII-like	BIII_406C	BIII
31	ND142	AI_A1	AI_A1	AI
32	ND145	BIII-like	-	BIII
33	ND153	BIII	BIII	BIII
34	ND156	BIII/BIII-like	BIII	BIII
35	ND166	AII_A2	BIV	AII+BIV

36	ND173	BIV-like	-	BIV
37	ND179	AII_A2_52T_445A	AII_A2	AII
38	ND182	BIV	BIV/BIV-like	BIV
39	ND196	BIV/BIV-like	BIV	BIV
40	ND201	BIII	BIII-like	BIII
41	ND205	AI_A1	AI_A1	AI
42	ND211	BIV-like	BIV	BIV
43	ND235	AI_A1	AI_A1	AI
44	ND243	BIII	BIII	BIII
45	ND250	BIII/BIII-like	BIII	BIII
46	ND258	AII_A2	AII_A2	AII

5.3.4. Phylogenetic relation of the isolates

Phylogenetic analyses conducted using the Maximum Likelihood (ML) method demonstrated that all sequences obtained in this study, at the *tpi* and *gdh* loci, exhibited well-resolved topologies presenting distinct clusters for assemblages A and B, alongside the reference sequences sourced from GenBank. A concatenated phylogenetic tree was constructed to gain insights into the relative positions of the isolates in relation to one another (Fig 5.6.). The mixed assemblage samples, namely ND4, ND41, ND83, and ND166, formed a distinct cluster located in the middle of the phylogenetic tree. These findings support the accurate grouping and alignment of the sequences within their respective genetic contexts.

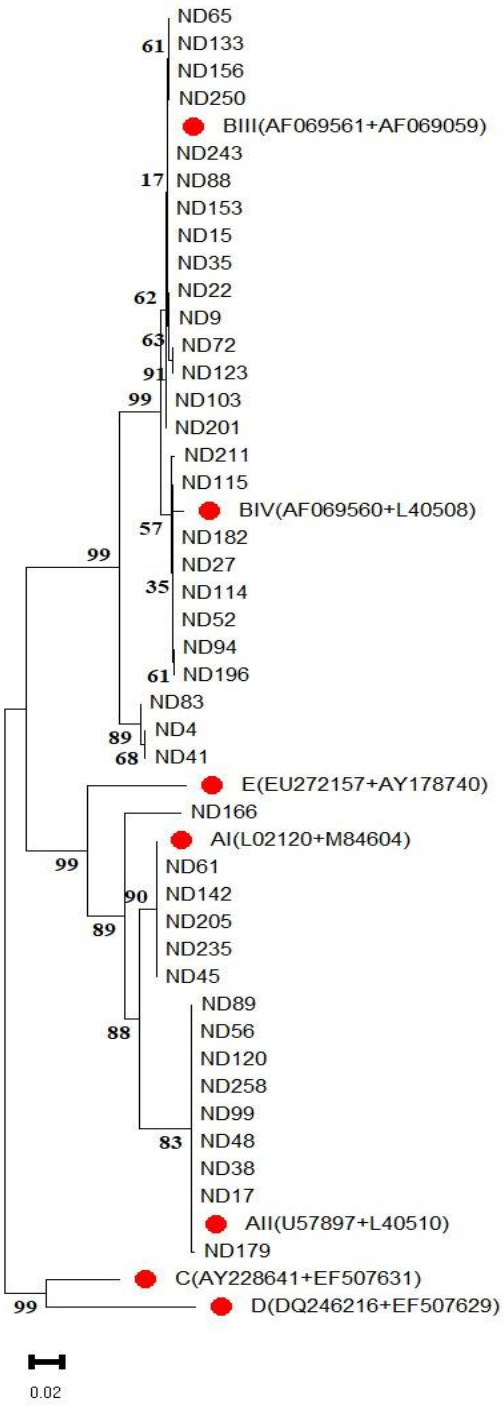


Fig 5.6. Concatenated phylogenetic tree of the study isolates based on nucleotide sequences of *tpi* and *gdh* loci

5.3.5. Haplotype variability and population genetics

Regarding the *tpi* locus of assemblage A, a total of 4 sites were found to be polymorphic, with 3 sites being singleton variable sites and 1 site being a parsimony informative site. This led to the identification of 4 haplotypes from the 17 *G. lamblia* sequences isolated from the study area. The average number of nucleotide differences (k), nucleotide diversity (π) and haplotype diversity (Hd) obtained for the sequences were 1.368, 0.002 and 0.640, respectively. Notably, in the present study, haplotype 1 (H1) was the most prevalent haplotype from the study area, occurring in the majority of the *G. lamblia* sequences. In the case of assemblage B, a total of 14 sites were polymorphic, with 3 sites being singleton variable sites and 11 sites being parsimony informative sites. This analysis led to the identification of 14 haplotypes from the 38 *G. lamblia* sequences isolated from the study area. Consequently, the average number of nucleotide differences (k), haplotype diversity (Hd), and nucleotide diversity (π) were 3.299, 0.875, and 0.00673, respectively (Table 5.3.). For assemblage B, haplotype 3 (H3) was the most prevalent haplotype observed in the study area (Fig 5.7.).

For the *gdh* sequences of assemblage A, a total of 3 sites were polymorphic, with all 3 sites being parsimony informative sites. This analysis revealed the presence of 3 haplotypes among the 15 *Giardia lamblia* sequences examined. Consequently, the average number of nucleotide differences (k), haplotype diversity (Hd), and nucleotide diversity (π) were 1.200, 0.629, and 0.0031, respectively. Within assemblage A, haplotype 1 (H1) was identified as the most prevalent haplotype in the study area. For the assemblage B sequences, a total of 11 sites were polymorphic, with 1 site being a singleton variable site and 10 sites being parsimony informative sites. This analysis revealed the presence of 10 haplotypes among the 27 sequences examined. Consequently, the average number of nucleotide differences (k), haplotype diversity (Hd), and nucleotide diversity (π) were 4.125,

0.872, and 0.01066, respectively (Table 5.3). Within assemblage B, haplotype 1 (H1) was identified as the most prevalent haplotype in the study area (Fig 5.8.).

Table 5.3: Summary table of intragenic haplotype variability of *G. lamblia* isolates at each locus

Loci	**Population**	**N**	**S**	**H**	**Hd (S.D.)**	**Π (S.D.)**	**K**	**LD |D'|**	**Rm**	**FS (*P*)**	**D (*P*)**
tpi	Assemblage A	17	17	4	0.640±0.073	0.00279±0.00044	1.368	Y = 1.0000 + 0.0000 X	0	0.536 (0.265)	0.477 (> 0.10)
	Assemblage B	29	38	14	0.875±0.036	0.00673±0.00053	3.299	Y = 0.9351 + 0.2121 X	2	-3.673 (0.015*)	-0.03189 (> 0.10)
gdh	Assemblage A	15	15	3	0.629±0.086	0.0031±0.00051	1.20	1.0000 +	0	1.338	0.894(> 0.10)

								0.0000 X		(0.275)	
	Assembl age B	2 4	2 7	1 0	0.872±0.0 38	0.01066± 0.00087	4.12 5	Y = 0.9182 - 0.0216 X	3	-0.566 (0.151)	1.467 88 (> 0.10)

N = number of samples, *S* = number of sequences, *H* = haplotypes, Hd = haplotype diversity, Π = nucleotide diversity, *K* = average, LD= linkage disequilibrium, R_m= Number of recombination events, FS = Fu's Fs, *D* = Tajima's D, S.D. = standard deviation, *P* = *P* value

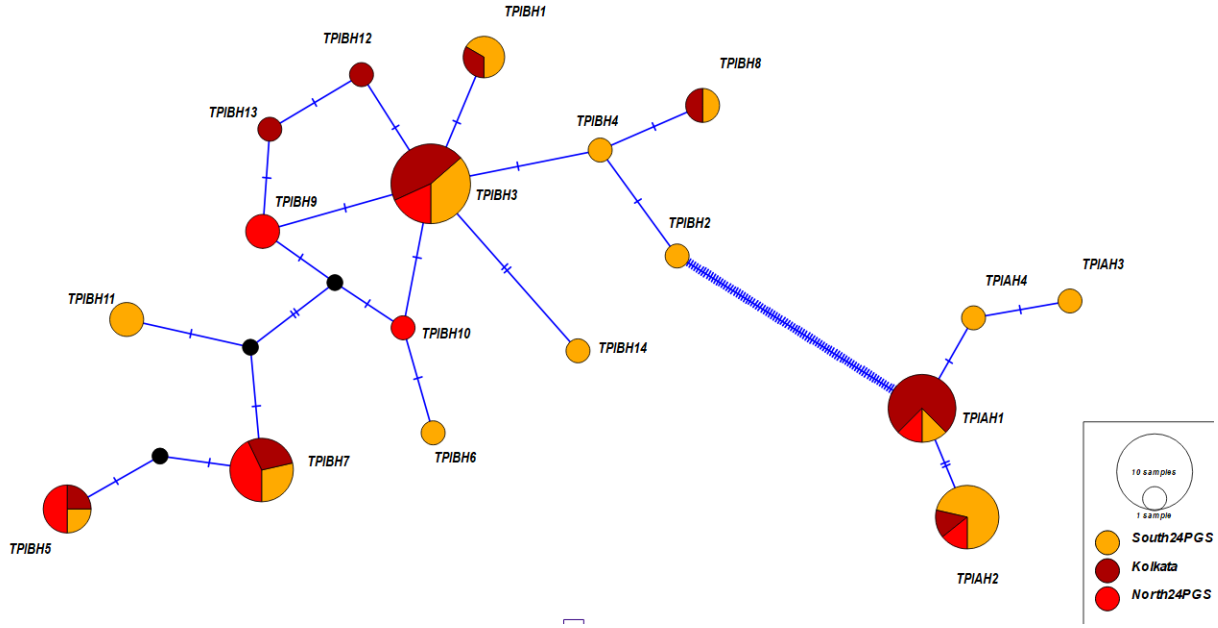


Fig 5.7. The median-joining haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the triose-phosphate isomerase (*tpi*) gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

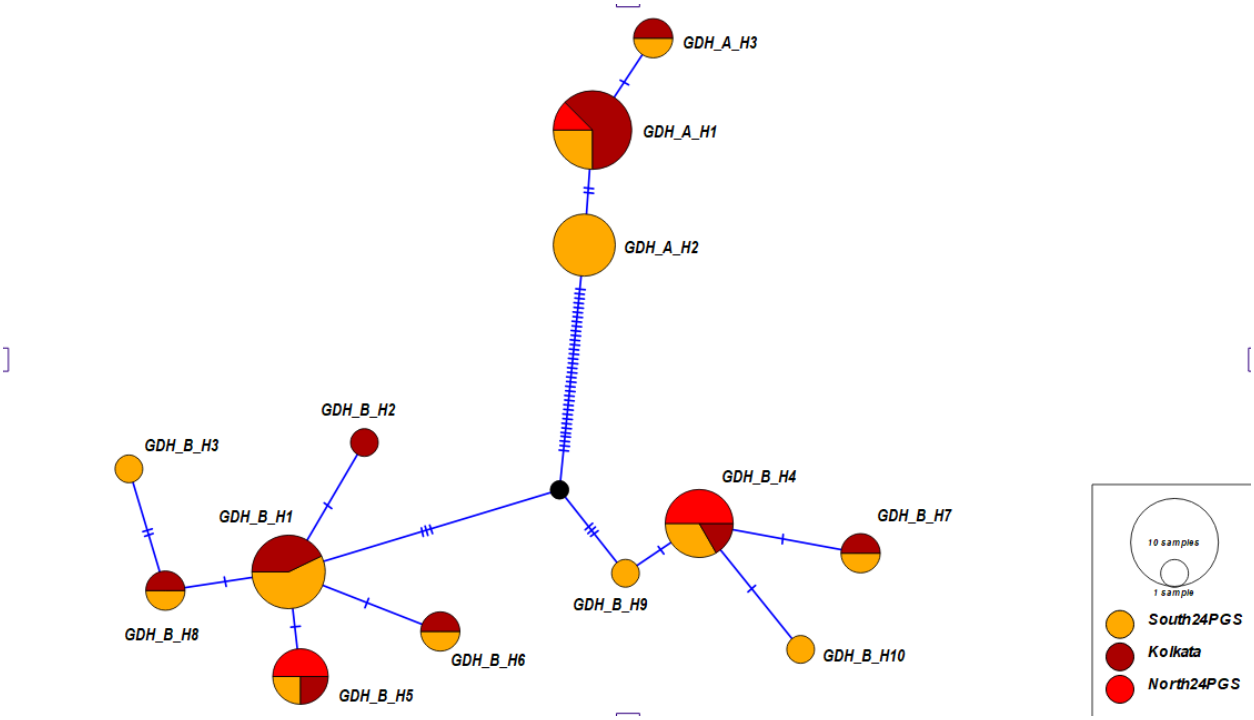


Fig 5.8. The median-joining haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the glutamate dehydrogenase (*gdh*) gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

The intragenic linkage disequilibrium (LD) and the number of potential recombination events were evaluated for each target locus. Incomplete intragenic LD values ($|D'|_{tpi}$ Y = 0.9351 + 0.2121X; $|D'|_{gdh}$ Y = 0.9182 - 0.0216X, where Y is the LD value and X is the nucleotide distance in kilobases) were identified at both loci for assemblage B. In contrast, a complete LD value (|D'| Y = 1.0000 + 0.0000X) was found at both *tpi* and *gdh* loci for assemblage A. The intragenic recombination test identified 2 potential intragenic recombination events (Rm) at the *tpi* locus and 3 events at the *gdh* locus for assemblage B. No recombination events were implicated at both loci for assemblage A (Table 5.3).

5.4. Discussion

This survey sheds light on the epidemiology of *G. lamblia* in indigenous communities of West Bengal. The key findings are: (i) giardiasis is predominant among apparently healthy individuals compared to symptomatic individuals in the same region (as indicated in hospital based survey), with the highest incidence in children aged 2–10 years; (ii) assemblage B is responsible for nearly 57% of the asymptomatic infections identified; and (iii) there is significant genetic heterogeneity within assemblage B sequences, irrespective of the molecular marker used, whereas assemblage A sequences display less diversity. Previous studies have also indicated that assemblage B is more prevalent than assemblage A in the region (Ghosal et al., 2023; Mukherjee et al., 2013a) and that assemblage B exhibits greater genetic variability (Koster et al., 2021; De Lucio et al., 2016). Several epidemiological studies conducted in endemic regions worldwide have reported a lack of positive correlation between *Giardia* infections and diarrhoea. These findings support our observation of a higher prevalence rate of *G. lamblia* in asymptomatic individuals, underscoring the fact that asymptomatic giardiasis is more common than symptomatic cases in certain environments (Bartelt and Platts-Mills, 2016; Koster et al., 2021).

Our study demonstrated that *G. lamblia* infection is significantly associated with younger age, seasonality, lower education level and socioeconomic status. These associations may be attributed to external factors linked to indirect transmission pathways, such as hygiene practices (e.g., consumption of contaminated fresh produce, swimming or bathing in contaminated surface waters, and high density of companion or domestic animals) or an increased risk of reinfection within the community through direct person-to-person contact. Contact with fecal contaminated water and

produce is more likely during the rainy season. Similar findings were reported in a study on asymptomatic tribal populations from the Brazilian Amazon (Koster et al., 2021).

To further investigate, multilocus genotyping (MLG) and sequencing were conducted on the positive *G. lamblia* isolates obtained from asymptomatic individuals. This analysis aimed to identify the infective assemblages/sub-assemblages and assess the genetic diversity among the relevant sequences. Microscopy-positive samples were subtyped using an MLG scheme targeting the triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes. Successful amplification and sequencing were achieved for 100% of the samples at the *tpi* locus and 89% at the *gdh* locus. However, MLG data for both loci were available for only 41 samples. The study identified BIII as the most prevalent sub-assemblage, followed by AII. This finding aligns with the results from a previous hospital-based survey conducted in the same study region, which outlined the relative proportions of various sub-assemblages and elucidated the prevailing trend of circulating *Giardia* assemblages in the area.

Sequences distinctly classified as sub-assemblages BIII and BIV at the *tpi* and *gdh* loci showed variability at one to six specific positions, termed hotspots, which appeared as either mutations or ambiguous (double peak) sites. In these hotspots, the proportion of double peak sites varied within assemblage B sequences, complicating the assignment of these ambiguous sequences to a particular sub-assemblage. Two potential mechanisms have been proposed to account for these ambiguous sites.

The first mechanism posits the presence of true mixed infections (e.g., A+B or BIII + BIV), which aligns well with an epidemiological scenario characterized by high rates of infection and reinfection, as observed in this study. The second mechanism involves genetic recombination

theory. Support for this comes from research indicating low levels of allelic sequence heterozygosity within assemblage B, suggesting a genetic homogenization process (Teodorovic et al., 2007). Additional evidence for genetic recombination has been found in studies showing recombination within assemblage B in single trophozoite and cyst cells (Ankarklev et al., 2012), as well as between sub-assemblages BIII and BIV at the population level (Koster et al., 2021; Siripattanapipong et al., 2011).

Intragenic linkage disequilibrium (LD) analyses revealed potential recombination events exclusively within assemblage B. The presence of numerous single nucleotide polymorphisms (SNPs), along with potential recombination events at both loci and negative values of Fu's Fs and Tajima's D, indicates a higher-than-expected diversity of haplotypes under neutrality. This pattern is typically associated with a recent population expansion under selective pressure. These findings collectively suggest that assemblage B has recently undergone demographic expansion driven by selective pressures. Previously, we observed that the assemblage B population isolated from diarrheal patients was under balancing selection pressure (Ghosal et al., 2023).

The study has several limitations. Firstly, certain independent variables such as the source of drinking water, garbage disposal location, defecation area, and handwashing practices were not included alongside other independent variables considered. Secondly, suspected mixed infections were not further investigated using cloning of PCR amplicons or next-generation sequencing. These methods offer high sensitivity in detecting genetic variants of the parasite that may be underrepresented in the population pool and otherwise undetectable using conventional PCR methods and Sanger sequencing. Lastly, the typing scheme utilized in this study may lack

sufficient phylogenetic resolution to accurately distinguish between sub-assemblage BIII and BIV sequences.

5.5. Conclusion

This pioneering MLG study marks the first genetic characterisation of human isolates of *G. lamblia* from asymptomatic communities in West Bengal. Our MLG analysis unveiled a higher prevalence of assemblage B in the population, particularly BIII, compared to assemblage A, suggesting an association of assemblage B with asymptomatic infection. Among assemblage A, the predominance of sub-assemblage AII in the studied individuals supports the notion that most human infections are acquired through anthroponotic sources, although zoonotic transmission of assemblage AI is also common. Population structure analysis indicated recent population expansion within the assemblage B. However, the typing scheme employed in this study lacks sufficient phylogenetic resolution to accurately differentiate between sub-assemblage BIII and BIV sequences. This underscores the imperative of identifying new markers and developing innovative methods for MLST purposes.

Chapter 6

DEVELOPMENT OF A RAPID AND COST-EFFECTIVE MULTIPLEX PCR-RFLP METHOD FOR GENOTYPING OF *Giardia lamblia*

6.1. Introduction

The occurrence rate of giardiasis varies across different populations, with developed countries reporting as low as a 2% prevalence, while developing countries exhibit a substantially higher occurrence, reaching up to 70% (Zajaczkowski et al., 2021). Globally, the annual occurrence of symptomatic giardiasis is estimated to be around 280 million cases (Einarsson et al., 2016). Numerous studies propose that asymptomatic infections are similarly prevalent, and the true global count of *G. lamblia* infections is probably much higher, given the underreporting resulting from the absence of symptomatic manifestations (Zajaczkowski et al., 2021). In preceding chapters, we have demonstrated that the prevalence among the asymptomatic population in the southeastern part of West Bengal is nearly double compared to what is observed in symptomatic cases of giardiasis in this region. This parasite is particularly prevalent among children, leading to severe intestinal disorders and stunted growth (Thompson & Monis, 2004). *G. lamblia* is recognized as a species complex and comprises eight morphologically similar assemblages, designated as A to H (Dos Reis et al., 2022). Assemblages A and B are identified as zoonotic genotypes because they have a broad host range and are found in other mammals besides humans (Adam et al., 2013). These genotypes share morphological similarities and are challenging to differentiate through microscopic examination. To genotype them, three well-conserved housekeeping genes—glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and triose phosphate isomerase—are analyzed for subtyping of *G. lamblia* (Cacciò & Ryan, 2008). However, conflicting genotyping results may arise when these genes are used independently, especially in samples involving mixed assemblages (Zajaczkowski et al., 2021). This challenge was encountered in the previous chapter while genotyping local isolates of *G. lamblia*. Additionally, despite advancements in genetically characterizing *G. lamblia*, there remains ambiguity regarding the associations with specific

symptoms and prevalence rates for assemblages A and B. To streamline the genotyping and subtyping process for *G. lamblia*, a rapid, cost-effective method is needed to enable simultaneous identification and differentiation between assemblage A and B. With this objective in mind, we conducted this study to develop a multiplex PCR-RFLP method, enabling the simultaneous detection and assemblage assignation.

6.2. Material and methods

6.2.1. *Giardia* Reference Strain and source of Genomic DNA

The *G. lamblia* assemblage AI, Portland-1 (ATCC-30888^{TM}) strain was obtained from the American Type Culture Collection (Maryland, USA) and axenically cultivated in the laboratory using modified TYI-S-33 medium, as described elsewhere (Keister, 1983).

6.2.2. Ethics and sampling

A total of 565 patients with diarrhea were enrolled in the study from April, 2023 to September, 2023. Fecal samples were collected from Infectious Disease & Beliaghata General Hospital and Dr. B. C. Roy Post Graduate Institute of Paediatric Sciences. The samples were then immediately transferred to the laboratory for processing. Prior to enrollment, informed consent was obtained from all adult patients and caregivers of the child patients, in accordance with the Institutional Ethics Committee guidelines by ICMR-NICED (IRB Number: A-1/2015-IEC).

6.2.3. Sample preparation

The fecal samples were divided into two aliquots. The first aliquot was utilized for microscopic examination following formol-ether concentration technique (Allen & Ridley, 1970). The second aliquot was immediately processed for DNA isolation.

6.2.4. Microscopy

To identify *G. lamblia*, the concentrated fecal samples were stained with iodine. Each sample was meticulously examined under a high dry (40×) objective of microscope (CX41, Olympus, Tokyo, Japan) in triplicate (Won et al., 2015). The presence of cysts and trophozoites was recorded.

6.2.5. Genomic DNA extraction

Genomic DNA of *G. lamblia* assemblage AI, Portland-1 strain was extracted from axenic culture using QIAamp DNA Minikit (Qiagen, USA) following the manufacturer's protocol. The genomic DNA of sub-assemblage AII and B, was generously provided by Prof. Seiki Kobayashi from NIID, Japan. DNA from the fecal samples was extracted using QIAamp DNA Stool Mini Kit (Qiagen, USA) following the manufacturer's protocol. The DNA was stored at -20°C until further use.

6.2.6. Primer design

Full-length *G. lamblia tpi* gene sequence (774bp) was selected to design specific primers for multiplex PCR assay. All the *tpi* sequences [*Giardia* Assemblage AI isolate WB (GL50803_93938); *Giardia* Assemblage A2 isolate DH (DHA2_93938) and *Giardia* Assemblage B isolate GS_B, (GSB_93938)] were obtained from the GiardiaDB database (https://giardiadb.org/giardiadb) and aligned using the ClustalW program (https://www.genome.jp/tools-bin/clustalw). Conserved regions among assemblage A and B were used for designing of a common forward primer GltpiMF- CCCTTCATCGGCGGTAACTT. Specific reverse primers (GltpiMR_A- GCCTTACAACACGCTTAGCCACA and GltpiMR_B- CACCTTCTGCGCAAACCTTT) were designed to selectively amplify both assemblages, resulting in distinct band sizes. The length, GC content and melting temperature (Tm) of all the

primers were optimized. The unique specificity of the primers was confirmed by Megablast program of the Basic Local Alignment Search Tool (BLAST) in the NCBI database.

6.2.7. PCR amplification

The optimal annealing temperature for the designed primer set was determined to be 57°C. The primer concentration of 0.2µM each was found to be optimal. The extension temperature was kept at 72°C to achieve the highest rate of amplification. The PCR reaction (25µl) consisted of approximately 2µl of template DNA, 1X buffer with 1.5mM $MgCl_2$ (TaKaRa, Japan), 200µM of dNTP (TaKaRa, Japan), 0.2µM of each primer (Eurofins, Luxembourg) and 2.5U of ExTaq (TaKaRa, Japan), along with nuclease-free water. The PCR products were resolved through 1.5% agarose gel electrophoresis and visualized under UV.

To assess the efficiency of the assemblage-specific primers, the results of the multiplex PCR amplification of clinical samples, were compared with a reference PCR method. The reference PCR utilizes a previously established nested protocol and conventional genotyping primer pairs against *tpi* locus. It amplifies a 531bp gene fragment for both A and B assemblages of *G. lamblia*.

6.2.8. Sequencing and Phylogenetic Inference

To confirm the assemblages, purified PCR products of differing lengths were subjected to bidirectional Sanger-dideoxy sequencing, using ABI 3730 PRISM® DNA Analyzer (Applied Biosystems, USA). The obtained sequences were aligned with reference sequences (assemblage A: L02120, AB569403, DHA2_93938, U57897; assemblage B: AB569404, AB569405, AF069560, AF069561, GSB_93938) and subsequently deposited in NCBI GenBank. A phylogenetic tree was constructed with MEGA 6.0 software using Tamura Nei parameter. Tree

robustness was assessed using the bootstrap method with 100 pseudo-replicates and values greater than 50% were reported.

6.2.9. PCR performance assay

To determine the specificity of the primers, we utilized DNA from stool samples positive for commonly encountered parasites, including *Entamoeba histolytica, Entamoeba moshkovskii, Entamoeba dispar, Cryptosporidium parvum, Cryptosporidium hominis, Cyclospora cayetanensis*, as well as bacterial pathogens viz., *Vibrio cholerae, Escherichia coli, Campylobacter jejuni, Salmonella* spp*., Shigella* spp. To compare the results, we incorporated *G. lamblia* positive fecal DNA samples and negative fecal DNA samples spiked with reference DNA of *G. lamblia* assemblage A and B. To assess the reliability of the method, 70 fecal DNA samples (of which 35 are *Giardia*-positive and genotyped in prior study) were included.

A sensitivity assay for the primer set was conducted in duplicate by serially diluting the reference DNA templates of both *G. lamblia* assemblage A and B. Serial dilutions were prepared with a five-fold gradient, using nuclease-free water with *Giardia* DNA, resulting in concentrations of 25ng/µL, L2: 5ng/µL, L3: 1ng/µL, L4: 200pg/µL, L5: 40pg/µL, L6: 8pg/µL, L7: 1.6pg/µL, L8: 0.032pg/µL, L9: 0.06pg/µL, L10: 0.01pg/µL, L11: 0.002pg/µL, L12: 0.0004 ng/µL. The lower detection limit (LDL) was elucidated and the concentration was converted to estimated copy number/µL using the following formula-

(https://www.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number)

$$\text{number of copies (molecules)} = \frac{\text{X ng} * 6.0221 \times 10^{23}\ \text{molecules/mole}}{(\text{N} * 660\ \text{g/mole})^{\dagger} * 1 \times 10^{9}\ \text{ng/g}}$$

Where:

X= amplicon amount (ng)

N = dsDNA length

660 g/mol = average mass of 1bp dsDNA

6.023 x 10^{23} = Avogadro's constant

1 x 10^{9} = Conversion factor

6.2.10. Restriction Fragment Length Polymorphism (RFLP) analysis

To identify subtypes within Assemblage A, the PCR product was subjected to digestion with *RsaI* enzyme (Takara, Japan), which recognizes the specific palindromic site GTAC. This site is present 3 times in the amplified fragment for AI and 4 times for AII. To subtype assemblage B, a double digestion was performed using *XhoI* and *BstBI* in Tango buffer (Thermofisher Scientific, USA). *XhoI* recognizes CTCGAG and induces a staggered cut within the sequence. Similarly, *BstBI* also creates a staggered cut within its recognition site TTCGAA. Following the digestion, the BIII subtype would generate 2 distinct fragments whereas, the BIV subtype would yield 3 separate fragments. All the expected fragments, theoretically generated, are given in Table 6.1. The restriction digestion reactions were carried out using 5U of each restriction enzyme in a total reaction volume of 30µL. To prepare the reaction mixture, ≈0.5µg of the amplified PCR product was combined with 3µL of 10x Tango buffer and nuclease-free distilled water to achieve the final volume. The reaction mixture was then incubated at 37°C for 40 minutes, followed by deactivation at 80°C for 15 minutes. Subsequently, the digested products were separated on a 1.5% agarose gel

and visualized under a UV transilluminator. Fig 6.1. illustrates the schematic diagram of the developed method.

Table 6.1. Fragment size of PCR products generated by selected restriction enzymes

Subasssemblage	Restriction Enzyme	Enzyme Cut Site	Predicted Fragment Length (bp)	Obtained Fragment Length (bp)
AI	*RsaI*	5′...GT▼AC...3′ 3′...CA▲TG...5′	566, 122, 65, 32	566, 122, 65
AII			364, 202, 122, 65, 32	364, 202, 122, 65,
BIII	*XhoI* + *BstBI*	5′...C▼TCGAG...3′ 3′...GAGCT▲C...5′ + 5′...TT▼CGAA...3′ 3′...AAGC▲TT...5′	428, 174	428, 174
BIV			275, 174, 153	275, 174, 153

6.2.11. Statistical Analysis

Statistical analysis of the data was conducted using GraphPad Prism version 9.0.0 (CA, USA) software. Diagnostic sensitivity (Se), specificity (Sp), accuracy (Ac), positive predictive value (PPV) and negative predictive value (NPV) were evaluated against the combined gold standard test results. The concordance of the diagnostic assay outcomes with other tests was assessed using Kappa statistics (κ) at 95% confidence intervals (CI).

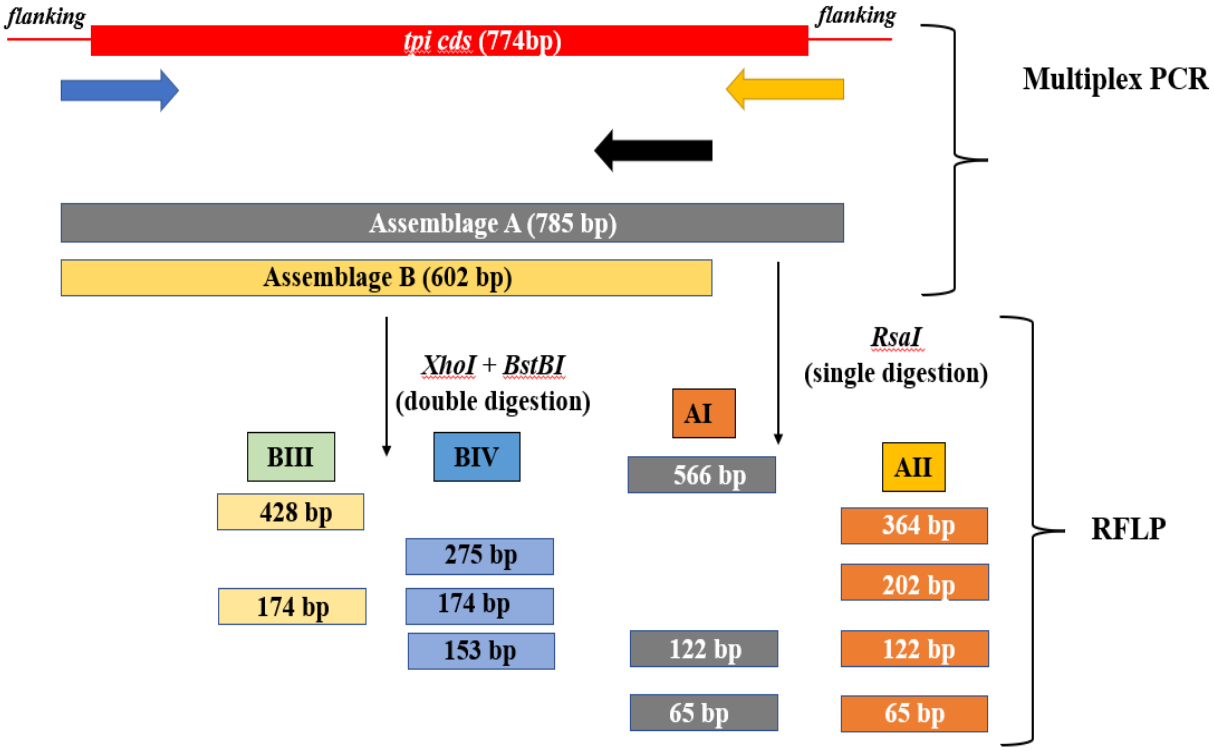


Fig. 6.1. Schematic diagram showing work flow of the developed multiplex PCR-RFLP method

6.3. Results

6.3.1. PCR amplification and restriction digestion

The optimized multiplex PCR protocol successfully amplified bands of predicted sizes from the *tpi* locus of reference strains of *G. lamblia*. It generated a 785bp amplicon for assemblage A and a shorter 602bp amplicon for assemblage B. When a mixed positive control DNA containing both assemblages (A+B) was used as a template, two distinct bands of the aforementioned sizes were observed (Fig. 6.2.).

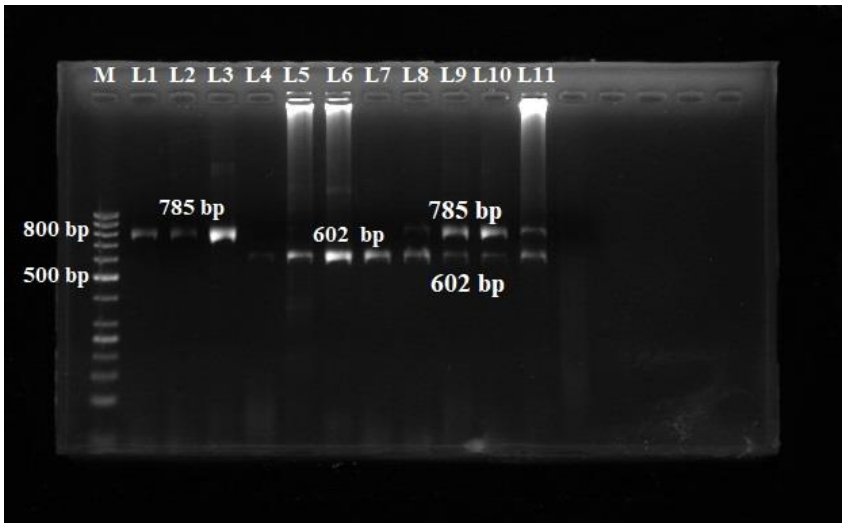


Fig. 6.2. PCR amplification products of G. lamblia-positive samples: M- 50 bp marker; L1-L2: *Giardia* positive stool sample (assemblage A)- exhibiting band at 785 bp; L3: Positive control DNA Portland 1 strain (assemblage A); L4-L6: *Giardia* positive stool sample (assemblage B) exhibiting band at 602 bp; L7: Positive control DNA (assemblage B); L8-L10: 3 mixed assemblage cases from previous genotyping study- mixed infection identified, band at both 785bp and 602 bp obtained; L11: Mixed positive control DNA of both assemblages (A+B)

The RFLP results obtained using the *RSaI* enzyme, with reference assemblage AI DNA (from the Portland 1 strain), exhibited three distinct DNA bands at 566, 122, and 65bp. Upon digestion of the PCR product from AII positive control DNA, four separate bands of 364, 202, 122 and 65bp

were produced. Similarly, double digestion of the PCR product from positive control DNA of assemblage B, with *XhoI* and *BstBI* enzymes generated 2 bands (428 and 174 bp) for BIII subassemblage and 3 bands (275, 174 and 153bp) for assemblage BIV (Fig. 6.3.).

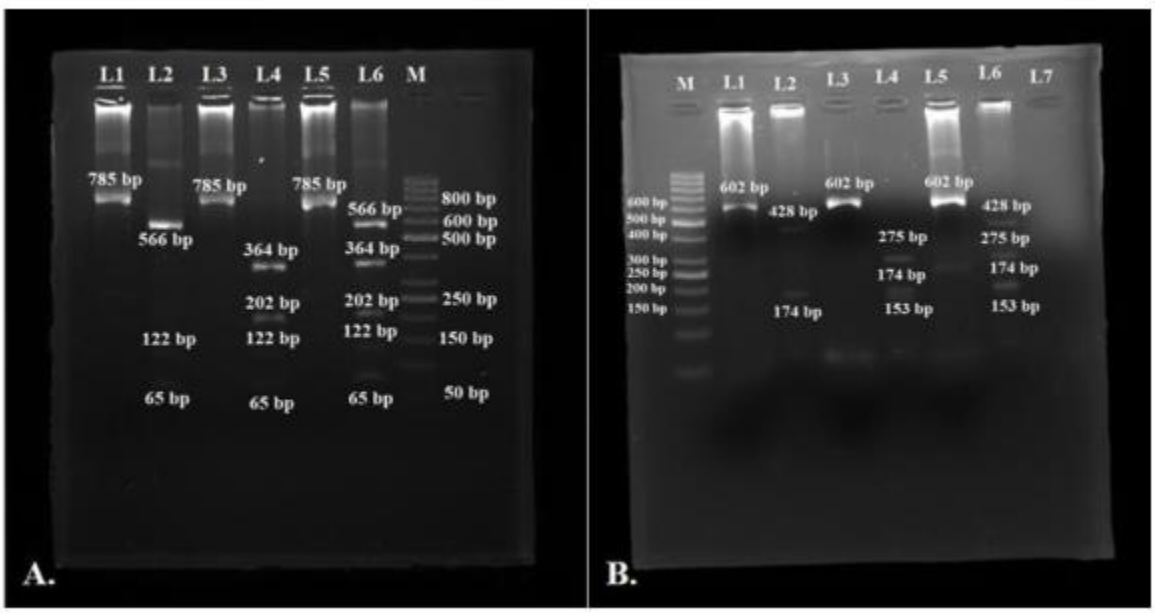


Fig 6.3. A. RFLP pattern of *G. lamblia* assemblage A isolates with *RsaI.* L1: Undigested PCR product, L2: Restriction profile for subassemblage AI (bands at 566, 122 and 65 bp), L3: Undigested PCR product, L4: Restriction profile for subassemblage AII (bands at 364, 202, 122 and 65 bp), L5: Undigested PCR product, L6: Restriction profile for mixed subassemblages AI+ AII (bands at 566, 364, 202, 122 and 65 bp), L7: 50bp marker. **B.** RFLP pattern of *G. lamblia* assemblage B isolates with *XhoI* and *BstBI.* M: 50bp marker, L1: Undigested PCR product, L2: Restriction profile for subassemblage BIII (bands at 428 and 174bp), L3: Undigested PCR product, L4: Restriction profile for subassemblage BIV (bands at 275, 174 and 153 bp), L5: Undigested PCR product, L6: Restriction profile for mixed subassemblages BIII+ BIV (bands at 428, 275, 174 and 153 bp)

6.3.2. Detection and Genotyping of *G. lamblia* in clinical Samples via PCR-RFLP method

The method was able to amplify the *tpi* gene, from two sets of fecal DNA samples: one set comprised previously genotyped DNA samples using the reference PCR method, while the other

set consisted of DNA extracted from newly collected clinical samples. The multiplex assay effectively amplified 33 out of 35 *Giardia*-positive control stool samples from the prior investigation. 16 of these samples were identified as assemblage A, produced bands at 785bp, while 14 samples classified as assemblage B, displayed band at 602bp. These findings were consistent with the previous genotyping results. Earlier 3 specimens generated incongruent results during reference genotyping PCR. The newly developed PCR assay successfully identified both A and B assemblages in them, indicating mixed infections (Fig. 6.2.). The 2 samples that failed to amplify, resulting in false-negative outcomes, were previously identified as positive using the reference PCR, that targets a 531bp region of the same gene. Subsequent RFLP analysis revealed that 9 samples belonged to Assemblage AII, while 7 samples correspond to Assemblage AI. Similarly, 7 samples were identified as Assemblage BIII, with another 7 categorized as BIV. In case of mixed infections, the identified genotype profiles were as follows: GLISO26 exhibited a genotype of AI+BIII, GLISO41 displayed a genotype of AII+BIII, and GLISO52 demonstrated a genotype of AI+BIV.

Out of the 565 new samples screened, 36 were found to be positive for *G. lamblia*, indicating a prevalence of 6.33% as determined by diagnostic PCR assay. Among these samples, 21 (58.33%) were identified as belonging to assemblage B, while 15 (41.66%) were classified as assemblage A. Of the 23 samples, 13 (36.11%) were identified as *G. lamblia* subassemblage BIII and 8 (22.22%) as subassemblage BIV. Among all assemblage A isolates, 5 (13.8%) were genotyped as subassemblage AI and 10 (27.77%) as subassemblage AII. No samples exhibited a mixed genotype. The *Giardia*-positive samples were sequenced to confirm the results. The obtained sequences were deposited in GenBank under accession numbers: PP584133-PP584205.

6.3.3. Phylogenetic analyses

The phylogenetic tree constructed with the obtained sequences showed a well-resolved tree topology, with distinct clusters corresponding to the A and B assemblages (Fig. 6.4.). The clinical isolates from this study were clustered within the appropriate subassemblage groups, alongside reference sequences and previously published sequences of the respective subassemblages. The concordance between the phylogenetic analysis and the PCR-RFLP results validates the ability of the method to accurately assign the isolates into their corresponding subassemblages.

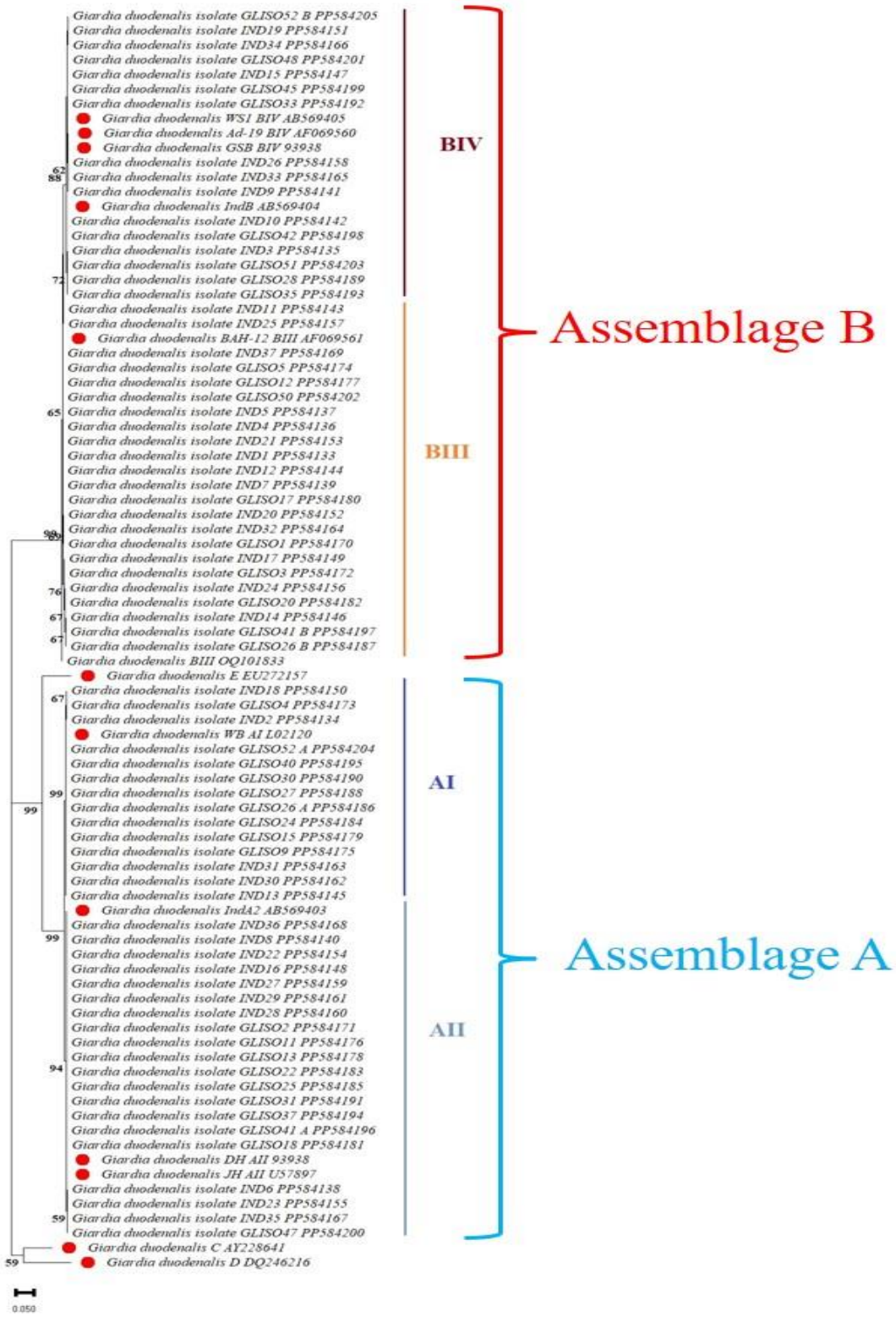


Fig 6.4. Constructed phylogenetic tree of study isolates based on nucleotide sequences of *tpi*

6.3.4. Evaluation of the Analytical performance of the PCR-RFLP assay

Although the PCR assay was capable of detecting and generating faint bands up to a concentration of 0.32pg/µl of genomic DNA (for both assemblages A and B), the estimated LDL for the assay was 1.6pg/µl (obtained in both runs), which corresponds to approximately 125 copies of the *Giardia* genome (Fig. 6.5.). However, this concentration proved insufficient to yield visible RFLP patterns for subtyping, thus providing information solely regarding assemblage. The analytical sensitivity of the RFLP assay, exhibiting differentiation of *Giardia* subassemblages, were as low as 0.2ng of genomic DNA for assemblage A and 5ng of genomic DNA for assemblage B per starting PCR reaction. These concentrations were able to produce easily discernible RFLP patterns after restriction digestion.

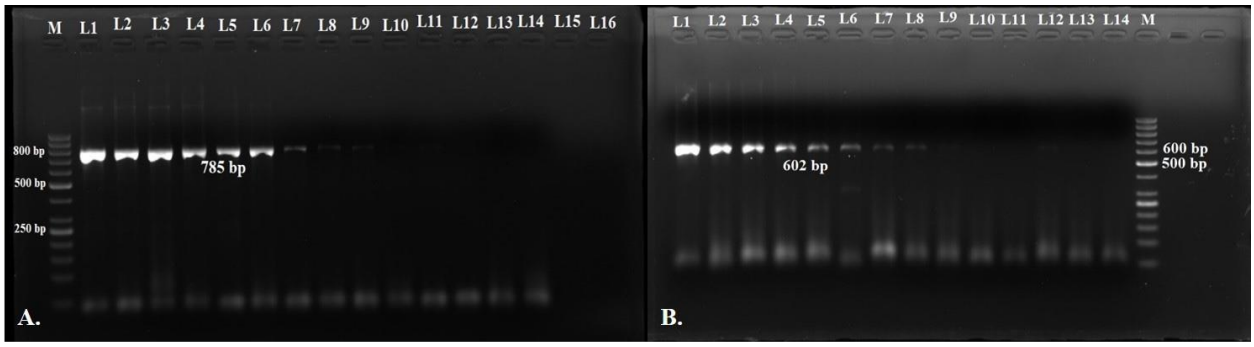


Fig 6.5. Determination of Lower Detection Limit (LDL) for assemblage A primer set: M – Marker/Ladder (50bp DNA Ladder), L1: 25 ng/µL, L2: 5 ng/µL, L3: 1 ng/µL, L4: 200pg/µL, L5: 40pg/µL, L6: 8pg/µL, L7: 1.6pg/µL, L8: 0.032pg/µL, L9: 0.06pg/µL, L10: 0.01pg/µL, L11: 0.002pg/µL, L12: 0.0004 ng/µL, L13: Negative control DNA, L14 – No template control. **B.** Determination of Lower Detection Limit (LDL) for assemblage B primer set: L1: 25 ng/µL, L2: 5 ng/µL, L3: 1 ng/µL, L4: 200pg/µL, L5: 40pg/µL, L6: 8pg/µL, L7: 1.6pg/µL, L8: 0.032pg/µL, L9: 0.06pg/µL, L10: 0.01pg/µL, L11: 0.002pg/µL, L12: 0.0004 ng/µL, L13: Negative control DNA, L14 – No template control, M –Marker/Ladder (50bp DNA Ladder)

6.3.5. Evaluation of diagnostic performance of the PCR assay

The designed primer set exhibited specificity in detecting the *tpi* sequence of *Giardia* origin. The assay successfully amplified DNA sequences from 33 out of 35 Giardia-positive control stool samples. Based on these findings, the diagnostic sensitivity (Se), specificity (Sp), accuracy (Ac), negative predictive value (NPV) and positive predictive value (PPV) of the PCR assay were determined to be 94.2%, 100%, 97.1%, 94.6% and 100%, respectively (Table 6.2.). No amplification was detected in any of the control stool samples that previously tested negative for *Giardia* (n=35). Furthermore, there was no observation of non-specific amplification in stool DNA positive for other protozoan and bacterial pathogens (Fig. 6.6.).

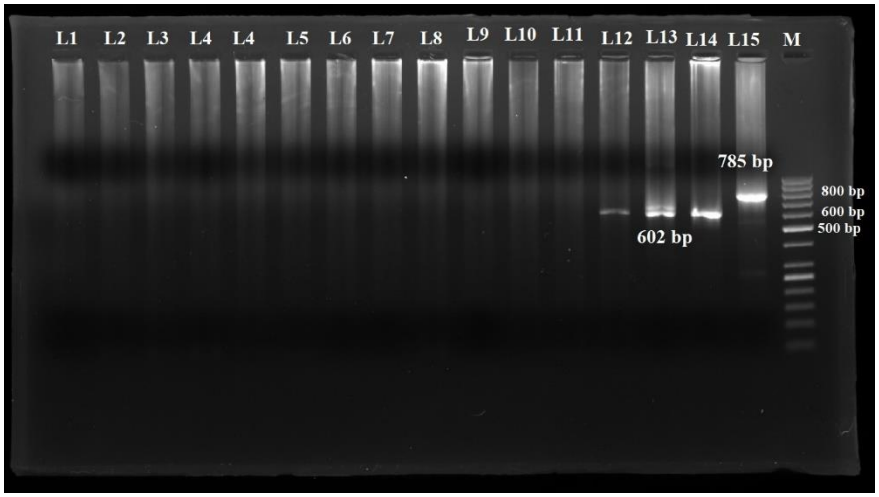


Fig 6.6. Specificity check of the multiplex PCR primer set with other enteric pathogens. L1: *Entamoeba histolytica;* L2: *Entamoeba dispar,* L3: *Entamoeba moshkovskii,* L4: *Cryptosporidium hominis,* L5: *Cryptosporidium parvum and* L6: *Cyclospora cayetanensis,* L7: *Vibrio cholerae,* L8: *Campylobacter jejuni,* L9: *Shigella* spp., L10: *Salmonella* spp. and L11: *Escherichia coli*; L12-14: *Giardia* positive stool DNA (assemblage B), L15: *Giardia* positive stool DNA (assemblage A)

Table 6.2. Diagnostic performance parameters

					Sensitivity (Se)		Specificity (Sp)		Accuracy (Ac)		Negative Predictive value (NPV)		Positive Predictive value (PPV)	
Test	True Positive (TP)	False Positive (FP)	True Negative (TN)	False Negative (FN)	%	95% CI	%	95% CI	%	95% CI	%	95% CI	%	95% CI
PCR assay	33	0	35	2	94.2	80.43-99.38	100	88.24-100	97.1	89.58-99.80	94.6	81.37-99.43	100	88.24-100

6.3.6. Concordance with other assay methods

A disparity was noted between the results of the reference PCR and the PCR-RFLP method during the screening of new clinical samples. The reference PCR identified *G. lamblia* DNA in 37 samples (6.54%), whereas the new PCR assay could only detect and differentiate 36 samples. *Giardia* cysts/trophozoites were detected via conventional microscopy in only 27 samples (4.77%). All samples that tested positive for *Giardia* via microscopy also tested positive with both PCR assays. The percentage of agreement between the PCR assay and other assay results is represented in Table 6.3.

Table 6.3. Concordance of results between the diagnostic PCR and other methods

	Results			Concordance			Cohen Kappa test	
Test	Positive	Negative	Prevalence (%)	Positive percentage agreement (PPA)	Negative percentage agreement (NPA)	Overall agreement (OA)	Kappa statistic (κ)	95 % CI
Microscopy	27	538	4.77	75	100	98.4	0.85	0.75-0.94
Reference PCR	37	528	6.54	97.2	100	99.8	0.98	0.95-1.00

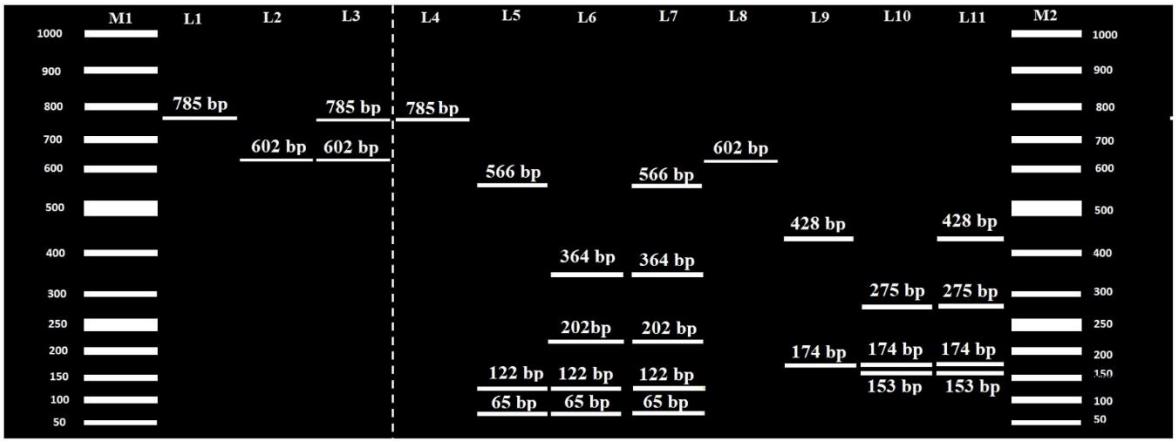


Fig 6.7. Schematic diagram showing all band patterns obtained after multiplex PCR and subsequent RFLP method

6.4. Discussion

The developed multiplex PCR-RFLP protocol has proven to be effective in accurately detecting and categorizing clinical isolates of *G. lamblia* into corresponding assemblages and subassemblages. This protocol generates distinctive PCR bands and unique RFLP band patterns for identification, offering a rapid and cost-effective option for clinical diagnosis of giardiasis, as well as for genotyping and epidemiological research. Fig. 6.7. depicts the schematic diagram of all the obtained band patterns.

Traditional microscopy-based detection for *Giardia* in stool samples require expertise, yielding results that may not always be accurate. Meanwhile, stool antigen-based immunodiagnostics offer improved accuracy and user-friendliness, compared to microscopy but has limited ability to ascertain genotypes/sub-genotypes effectively. Nucleic acid-based diagnostic methods like PCR, have gained traction in clinical investigations because of their improved sensitivity, specificity and capacity to yield genotype data. Compared to other methods, the accuracy of PCR is facilitated by the stability of its target material i.e., DNA (Chang et al., 2023).

In this study, to identify and genotype the *G. lamblia* isolates, the *tpi* gene was targeted. This particular gene was reported to be suitable for population genetics studies as it is highly informative and ample sequence data are available in public databases for comparative analysis (Nolan et al., 2010; Xiao & Feng, 2017). The efficacy of the *tpi*-based genotyping tool has been established in many epidemiological studies of giardiasis among human populations (Sulaiman et al., 2003). Multiple studies have employed this locus for PCR-RFLP technique (Rahimian et al., 2018; Nolan et al., 2010 Helmy et al., 2009; Torres-Romer et al., 2014) as this single copy gene exhibits significant genetic heterogeneity, rendering it an ideal marker with robust discriminatory

ability (Zajaczkowski et al., 2021; Sulaiman et al., 2003). The restriction sites and primer binding regions were carefully selected from the conserved segments among all the reported genotypes, to ensure the success of our assay. The genotyping results for the reference strains and clinical isolates exhibited congruence with the predicted outcomes, indicating the conserved nature of the selected sites, thereby validating the robustness of the assay.

This PCR-RFLP assay uses a set of assemblage-specific primers in the initial step to discern between human infecting *Giardia* assemblages. Multiplex PCR with assemblage-specific primers offers inherent advantages, allowing for the discrimination of various target sequences based on the lengths of PCR products (Zajaczkowski et al., 2021). It reduces turnaround time by eliminating the need for separate reactions per target and minimizes reagent and consumable costs compared to individual reactions. Moreover, it helps in genotype differentiation without sequencing procedures. A similar multiplex PCR method was described previously, utilizing the same locus, where four pairs of assemblage-specific primers differentiated between *G. lamblia* assemblages A and B. The procedure yielded a 476bp fragment for assemblage A, later subjected to RFLP analysis, leading to differentiation into two groups (groups I and II). For assemblage B, a much shorter 140bp fragment was amplified, which could not be further subtyped into subassemblages (Amar et al., 2002; Amar et al., 2003). Our method utilizes lesser number of primers to discriminate between the assemblages. This is a single-step process and hence consumes less time. Furthermore, the produced fragments for each assemblage are long enough to undergo RFLP technique to differentiate subassemblages.

Another PCR-RFLP method described utilizes general *tpi* primer pair in a nested PCR protocol and requires 3 steps to distinguish between the assemblages (Rahimian et al., 2018). Consequently,

it is time-consuming and requires more reagents compared to our method that consolidates these steps into a streamlined workflow.

The PCR assay exhibited significant analytical sensitivity (94.2%) with a high accuracy rate (97.1%), capable of detecting approximately 125 copies of the *tpi* gene from cultured trophozoites. A comparable PCR-RFLP approach targeting the *gdh* gene demonstrated an LDL of 2pg/µl of *Giardia* genomic DNA (Hawash et al., 2016). However, this method required post-PCR RFLP analysis for assemblage determination, again rendering it more time-consuming to achieve similar results. This approach can only classify isolates to the assemblage level, precluding sub-assemblage identification.

Our assay exhibited good specificity, with no cross-reactivity. The false-negative results obtained for the two samples compared to the reference PCR results are possibly due to low parasite load in the samples. The reference PCR uses a nested protocol, which may have aided in the detection of the low DNA concentration in those samples, which our single-step method failed to achieve. The distribution pattern obtained in this study for the *Giardia* assemblages in patients was consistent with previous reports from the region, showing a predominance of Assemblage B (Mukherjee et al., 2013). Although the sample size employed in this study was adequate for statistical analysis, further investigations with a larger sample size across broader geographical regions are warranted to obtain a more comprehensive evaluation of the specificity and sensitivity of this assay.

Compared to that of the reference PCR, this novel diagnostic method described a high level of agreement between them (κ=0.98). The outcomes of the PCR-RFLP method matched sequencing data, affirming specificity of the method.

However, this approach has a few limitations. It requires PCR products with high band intensity (>20ng/µl) to obtain clear RFLP patterns on agarose gel. Moreover, the RFLP method precludes identification of SNPs outside the restriction sites within the amplified region, thereby limiting comprehensive detection of sequence variations across the entire gene.

Nevertheless, this method was designed to be adaptable in resource-poor settings, requiring minimal equipment and expertise. It only requires conventional molecular equipment like thermal cycler and electrophoresis system, which are commonly present in any laboratory. Moreover, it takes approximately 4 hours to execute the detection and differentiation of *G. lamblia* in clinical samples, down to the subassemblage level. It can be suitable for processing large number of samples in outbreak investigations. Quick profiling of *G. lamblia* populations during epidemiological investigations can help with the identification of the source of infection. The method will prove to be beneficial in regions where routine screening is not conducted, resulting in underestimation of giardiasis cases, due to limited expertise or sophisticated instrumentation.

6.5. Conclusion

We designed a single-step PCR-RFLP assay to identify and differentiate *G. lamblia* clinical isolates from human fecal samples. The assay has been demonstrated to be highly specific, sufficiently sensitive and hence reliable. Additionally, it is time-efficient and cost-effective. The method shows considerable potential to be a diagnostic tool in clinical laboratories, especially in resource poor setting.

Chapter 7

GENETIC CHARACTERIZATION OF THE POTENTIAL VIRULENCE FACTORS OF THE *Giardia lamblia* LOCAL ISOLATES

7.1. Introduction

Giardia lamblia is an intestinal protozoan parasite of the order Diplomonadida that constitutes a species complex of eight different phylogenetic groups or assemblages (A-H), characterized by different host specificities (Thompson and Monis, 2012). These assemblages are defined based on genetic markers mentioned earlier. Assemblages A and B are of particular relevance to human public health as they are zoonotic, meaning they can be transmitted between animals and humans (Klotz et al., 2023). Additionally, Assemblages A and B may represent distinct species. Assemblages A and B were previously considered the most virulent ones, but the existence of asymptomatic carriers and considerable genetic variability within and among these assemblages makes it challenging to define common virulence factors (Garcia et al., 2021). The pathophysiological consequences of *Giardia* infection are multifactorial, involving a combination of host and parasite factors, as well as immunological and non-immunological processes occurring at the mucosal level (Ortega-Pierres et al., 2019).

The attachment of *Giardia* trophozoites to epithelial cells greatly depends on structural components of the adhesive disk, such as giardins or tubulins. Additionally, the secretomes of these trophozoites upon their interaction with specific epithelial cell lines such as Caco-2 cells, have identified a series of other potential virulence factors. These include glycolytic enzymes (e.g., enolase), arginolytic enzymes (e.g., arginine deiminase), cysteine proteases (e.g., giardipain-1), and variant-specific surface proteins (VSPs) (e.g., VSP9B10A), Tenascins and High Cysteine Membrane Proteins (HCMPs) (Garcia et al., 2021). In addition to the structural and secretory proteins, *Giardia lamblia*, an amitochondriate organism, possesses an array of metabolic enzymes like Pyruvate ferredoxin oxidoreductase (PFORs), Thioredoxin reductase (TrxR), Nitroreductase (NR), NADH ferredoxin oxidoreductase, Malate dehydrogenase etc., that aid in relieving the

oxidative and nitrosative stress encountered within the host gut environment, indirectly contributing to the adaptation and virulence of the parasite (Raj et al., 2014).

While giardiasis is often self-limiting, treatment is necessary to alleviate symptoms, reduce the risk of post-infection complications, and prevent the prolonged shedding of infectious cysts. Currently, there is no available prophylaxis for giardiasis, but several drugs are approved for its treatment, including 5-nitroimidazoles, benzimidazole derivatives, quinacrine, furazolidone, nitazoxanide, and paromomycin (Lalle, 2010). Among these, metronidazole or tinidazole are the most commonly used drugs in humans, but alternatives like furazolidone or paromomycin are also recommended (Spickler, 2012). Metronidazole (MTZ) is a prodrug, which needs to be intracellularly activated by partial reduction at its nitro group to create highly reactive toxic intermediates (Saghaug et al., 2023). MTZ has an estimated efficacy rate ranging from 60% to 100% (Argüello-García et al., 2020) in curing giardiasis. Over the past decade, treatment failures with MTZ have been reported more frequently from all over the world, raising concerns (Lalle and Hanevik, 2018). Prevalence of the refractory giardiasis, not cured with the standard 5-7day course of MTZ treatment, ranges between 15% to 70%. Many of such cases were reported from Indian subcontinent (Munoz Gutierrez et al., 2013; Nabarro et al., 2015; Requena-Mendez et al., 2017; Cañete et al., 2020; Ydsten et al., 2021; Neumayr et al., 2021). This highlights the growing issue of MTZ resistance in treating giardiasis. Several reports indicate that metronidazole (MTZ) resistance mechanisms in *G. lamblia* involve the downregulation of genes responsible for MTZ's cytotoxic effects or the upregulation of genes that inactivate MTZ (Krakovka et al., 2022, Ansell et al., 2017, Muller et al., 2019, Muller et al., 2008).

Isolates belonging to Assemblage B of *G. lamblia* typically exhibit higher genetic diversity and allelic sequence heterozygosity (ASH) compared to isolates from Assemblage A. Studies have

reported an ASH of approximately 0.5% in Assemblage B isolates, whereas Assemblage A isolates have shown lower ASH values ranging from <0.01% to 0.04% (Yu et al., 2002; Franzen et al., 2009; Ankarklev et al., 2012). The *Giardia* genome exhibits remarkable variability, and the genes contributing to virulence are not well documented. Hence, this study aims to investigate the genetic variability of local *Giardia* isolates concerning various virulence factors.

7.2. Materials and methods

7.2.1. Samples

The study utilizes a total of 45 samples from three different groups: 15 samples from diarrheal patients with a sole *Giardia* infection, 15 diarrheal samples with co-infections of *Giardia* and other enteric pathogens, and 15 asymptomatic samples that tested positive for *Giardia*. These specific samples were previously identified using a combination of microscopy and polymerase chain reaction (PCR) techniques targeting the beta-giardin (*bg*) locus, as described in earlier chapters.

7.2.2. PCR amplification

The positive samples were used to amplify target genes: NR1, NR2, ENO, ef-1α. The amplification process was carried in two fragments: both 25µL reaction mixture for PCR step containing 2.5 units of TaKaRa Ex-Taq polymerase, 1X PCR buffer, 0.2µM of both outer forward and outer reverse primers, and 3µL of stool DNA samples with a concentration of 5ng/µL.

The amplification reactions were performed using an Applied Biosystems thermal cycler PCR system. Both the PCR steps began with an initial denaturation at 95°C for 3 minutes, followed by 35 amplification cycles. Each cycle consisted of a denaturation step at 95°C for 30 seconds, an annealing phase at the determined annealing temperature for each gene for 30 seconds, a polymerization step at 72°C for 45 seconds and a final extension step at 72°C for 7 minutes.

Table 7.1. provides the primer sequences and annealing temperatures used for each gene. After amplification, the PCR products were subjected to 1% agarose gel electrophoresis (Seakem® LE Agarose, Lonza) and visualized under a UV transilluminator after staining with 0.5 μg/ml ethidium bromide.

Table 7.1. Primers, target loci, primer sequences with corresponding length and annealing temperature used in protocol.

Name	Locus	Sequence	Length (bp)	Annealing (°C)
NR2F1	NR2	5'-ATGGTTGAAGGTTATCC-3'	17	48
NR2R1		5'- TGCAGGTGCAGACATCAG-3'	18	
NR2F2		5'-CCGGAGGTCTCAAGAAATC-3'	19	48
NR2R2		5'-GGCTCACTTAAAGGTAAT-3'	18	
NR1F1	NR1	5'-ATGTCACGCTTTCCAGAGG-3'	19	53
NR1R1		5'-GTGCACCGTGAAAAATGGG-3'	18	
NR1F2		5'-GTTTGTGACTGCGCTATCG-3'	19	52
NR1R2		5'-TGTCACATCAGCGCCTTC-3'	18	
EFTF1	EF1a	5'-ATGGGCAAGGAGAAGAAGCAC-3'	21	57
EFTR1		5'-TTGTCGGTCGGGCGCTTC-3'	18	
EFTF2		5'-CCGACAAGATGCCCTGG-3'	17	53
EFTR2		5'-TCACTTGCCGCCCTTGGG-3'	18	
ENOF1	Enolase	5'-ATGGAGGCTCCGTCTACGAT-3'	20	50
ENOR1		5'-ATGTACTTGAGAGGAACCT-3'	19	
ENOF2		5'-ATCCTCAAGGACGAGTT-3'	17	48
ENOR2		5'-CTACTTCCAGGCATCGAA-3'	18	

7.2.3. DNA Sequencing

The PCR products of expected sizes were extracted using a Roche Gel Extraction Kit following the manufacturer's protocols. The yield of the purified PCR products was verified by gel electrophoresis. These purified products were then subjected to direct sequencing in both forward and reverse directions using the corresponding amplification primers. The sequencing process

employed the BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, USA. The obtained sequences were analyzed using an ABI3730 sequencer.

7.2.4. Sequence Analysis

The obtained DNA sequences were aligned using the ClustalW multiple sequence alignment program from GenomeNet Bioinformatics resources and edited manually. Both synonymous and nonsynonymous single nucleotide polymorphisms (sSNPs and nsSNPs) present in the local isolates were identified by aligning the sequences with reference sequences obtained from GiardiaDB using the MultAlin online tool. Representative nucleotide sequences of each haplotype were deposited in the NCBI GenBank. Relationships between haplotypes were inferred by constructing a minimal-spanning haplotype network using PopART v1.7, with isolates color-coded based on their infection type status. The evolutionary relationships among the sequences of each locus were determined using the maximum likelihood (ML) method. The Tamura-Nei parameter model in MEGA11 software was utilized to calculate the evolutionary distances. To evaluate the robustness of the resulting phylogenetic trees, the bootstrap method was applied with 100 pseudo-replicates. Only bootstrap values greater than 50% were reported, indicating the statistical support for the branching patterns observed in the trees.

7.3. Results

7.3.1. Amplification of the target loci

Successful amplification was achieved in only 31 samples across 4 designated target loci. Some samples exhibited faint amplification, insufficient for sequencing, while others showed no distinct bands upon agarose gel electrophoresis. The lack of amplification in certain samples could be due to either low DNA concentration in fecal samples or issues with DNA quality. Additionally, genetic

polymorphisms at the specified target sites could also contribute to the observed lack of amplification.

7.3.2. Synonymous and non-synonymous single-nucleotide polymorphisms

The full-length genes NR1, NR2, Enolase, and ef-1α from both assemblages A and B isolates were successfully amplified and sequenced to characterize single nucleotide variants (SNVs) and allelic variation. The overall genetic variation, defined as the total number of combined SNP positions for each assemblage per gene length, was as follows: For Assemblage A, NR1 had 0.47%, NR2 had 0.37%, Enolase had 0.37%, and ef-1α had 0.15%. For Assemblage B, NR1 had 3.11%, NR2 had 5.4%, Enolase had 2.98%, and ef-1α had 0.82%. The percentage of total SNPs for Assemblage A isolates was lower compared to Assemblage B isolates. Notably, an exceptionally high percentage of overall SNPs was found in the NR2 locus in Assemblage B, whereas NR1 and Enolase had similar percentages of overall SNPs. The percentages of synonymous SNPs were higher than nonsynonymous SNPs (nsSNPs) in both assemblage isolates. For Assemblage A, the NR1 locus had 0.23% nsSNPs, the NR2 locus had 0.25%, while no nsSNPs were found in the Enolase and EF1-a loci. In Assemblage B, the percentages of nsSNPs per CDS were also higher, with the NR1 gene harboring 1.7%, the NR2 gene harboring 1.38%, and lower percentages in Enolase and ef-1α, with 0.52% and 0.0001% respectively.

7.3.3. Phylogenetic Relation

Phylogenetic analyses using of sequences from this study Maximum Likelihood (ML) method, encompassing four loci (ef-1α, Enolase, NR1, and NR2), yielded well-resolved topologies. These trees distinctly clustered assemblages A and B, consistent with reference sequences from GenBank. Individual phylogenetic trees were constructed to elucidate the relative positioning of isolates with

reference strains (Fig. 7.1-7.4). The result corroborated the accurate classification and alignment of sequences within their respective genetic lineages.

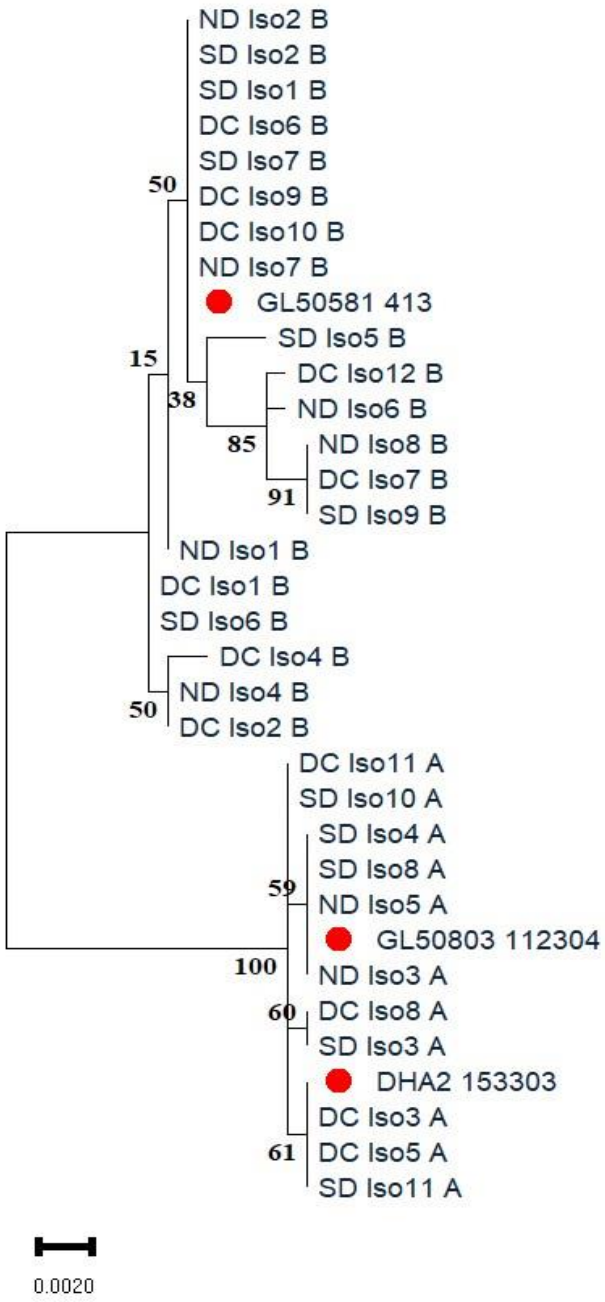


Fig 7.1: Phylogenetic tree of the study isolates based on nucleotide sequences of ef-1α

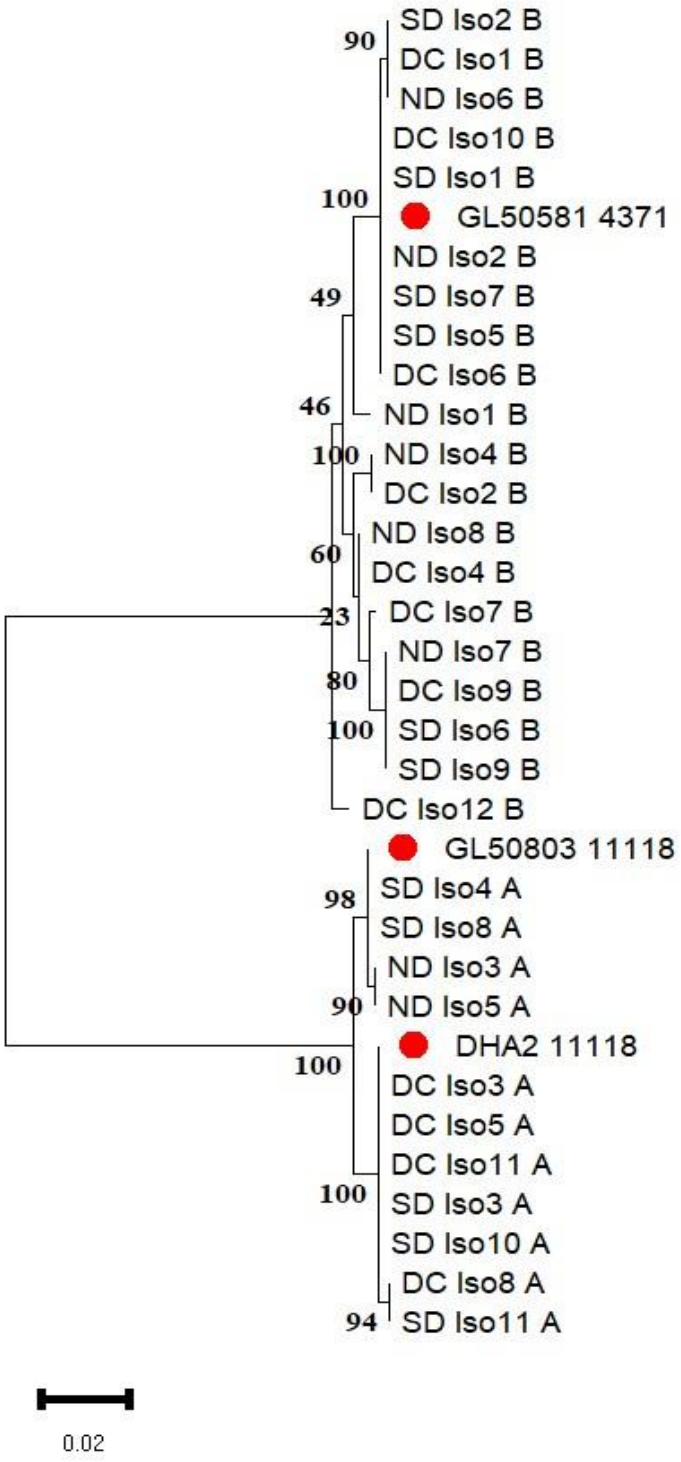


Fig 7.2: Phylogenetic tree of the study isolates based on nucleotide sequences of Enolase

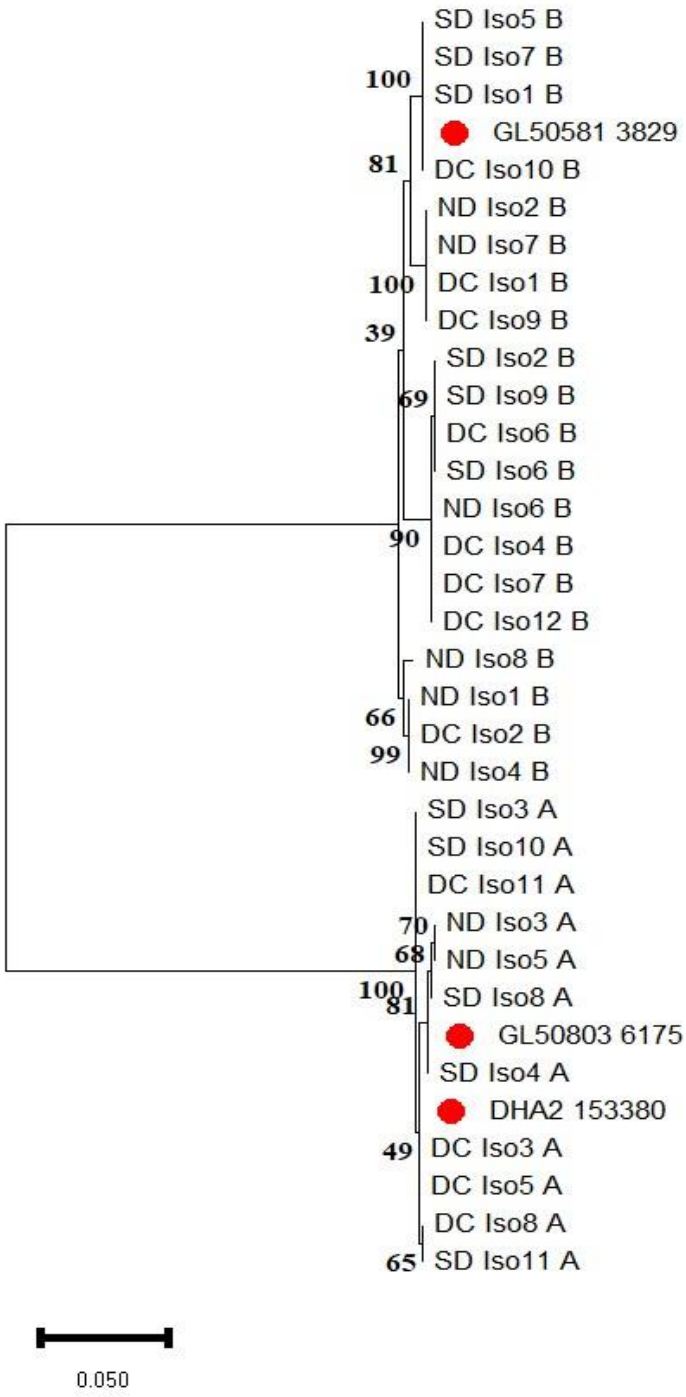


Fig 7.3: Phylogenetic tree of the study isolates based on nucleotide sequences of NR1

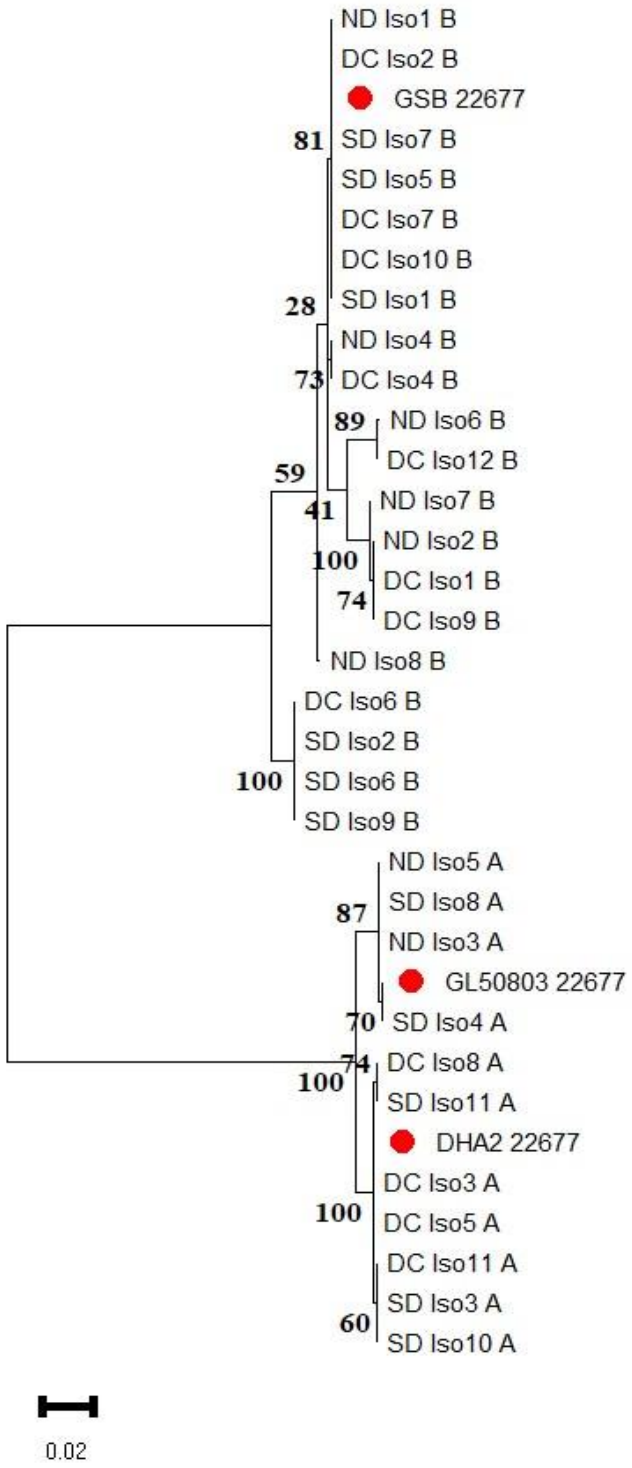


Fig 7.4: Phylogenetic tree of the study isolates based on nucleotide sequences of NR2

7.3.4. Genetic diversity

7.3.4.1. Assemblage A

Within the 11 samples of *G. lamblia* assemblage A, the number of haplotypes obtained was 4. Among the 4 examined loci, when calculated for all 3 groups together, the Hd values obtained for ef-1α, Enolase, NR1, and NR2 were 0.8, 0.764, 0.891, and 0.855, respectively. The nucleotide diversity (π) calculated for all groups together for all 4 loci was 0.00096, 0.0057, 0.00365, and 0.00904, respectively. The number of haplotypes (H) obtained for ef-1α, Enolase, NR1, and NR2 were 4, 4, 6, and 5. Therefore, the NR1 locus had the highest Hd value and the highest number of haplotypes. However, the NR2 locus exhibited the highest observed nucleotide diversity and the second-highest number of haplotypes and haplotype diversity. A low level of nucleotide diversity was observed for ef-1α, indicating its relatively conserved nature among the isolates, while Enolase and NR1 exhibited moderate nucleotide diversity. Among the examined loci, the Enolase locus showed the greatest number of polymorphic sites (17), while the ef-1α locus showed the fewest. For all 4 loci sole diarrheal group showed higher Hd value, π value and greater number of haplotype than other groups (Table 7.2).

7.3.4.2. Assemblage B

Within the 20 samples of *G. lamblia* assemblage A, 9 haplotypes were identified. For the 4 examined loci, calculated across all 3 groups, the Hd values for ef-1α, Enolase, NR1, and NR2 were 0.826, 0.863, 0.858, and 0.837, respectively. The nucleotide diversity (π) for all groups combined across the 4 loci was 0.00268, 0.00926, 0.0121, and 0.01862, respectively. The number of haplotypes (H) observed for ef-1α, Enolase, NR1, and NR2 were 9, 8, 6, and 8. For assemblage B isolates, the Enolase locus exhibited a relatively higher Hd value, although ef-1α had the highest

number of haplotypes. Ef-1α and Enolase loci showed relatively lower nucleotide diversity compared to NR1 and NR2, with NR2 displaying the highest nucleotide diversity. Among the examined loci, NR2 presented the greatest number of polymorphic sites (43), while ef-1α had the lowest. Regarding all 4 loci, the non-diarrheal group demonstrated higher Hd values, although a greater number of haplotypes was observed primarily in the coinfection diarrheal groups (Table 7.3).

Table 7.2: Summary table of intragenic haplotype variability of *G. lamblia* assemblage A isolates at each locus

Assemblage	**Locus**	**Population**	**N**	**S**	**H**	**Hd (S.D.)**	**Π (S.D.)**	**K**	**LD |D'|**	**Rm**	**FS (*P*)**	**FLD (*P*)**	**FLF (*P*)**	**D (*P*)**
A	ef-1α	All	11	11	4	0.80±0.075	0.00096±0.00012	1.273	Y = 1.0000 - 0.0000X	0	-0.22 (0.255)	1.127 (> 0.10)	1.081 (> 0.10)	0.83 (> 0.10)
		Sole-Diarr	5	5	4	0.9±0.161	0.001±0.00024	1.4	Y = 1.00	0	-1.6	-0.1	-0.1	-0.1

		heal (SD)							00 - 0.00 00X		48 (0. 142)	74 (> 0.1 0)	75 (> 0.1 0)	74 (> 0.1 0)
		Non-Diarr heal (ND)	2	2	1	0	0	0	NA	N A	NA	NA	NA	NA
		Diarr heal Coinf ection (DC)	4	4	3	0.833 ±0.22 2	0.0008± 0.0002	1. 16 7	Y = 1.00 00 - 0.00 00X	0	- 0.6 58 (0. 286)	0.5 91(> 0.1 0)	0.5 03 (> 0.1 0)	0.5 91 (> 0.1 0)
	Eno lase (EN OL)	All	1 1	1 1	4	0.764 ±0.09 9	0.0057± 0.001	7. 74 5	1.00 00 + 0.00 00X	0	5.0 22	1.5 3 (0.0 2*)	1.7 2 (0.0 2*)	1.4 97(>

											(0.03*)			0.10)
		Sole-Diarrheal (SD)	5	5	3	0.8±0.164	0.0062±0.001	8.4	Y = 1.0000 - 0.0000X	0	3.405 (0.179)	1.218(>0.10)	1.298(>0.10)	1.218(>0.10)
		Non-Diarrheal (ND)	2	2	1	0	0	0	NA	NA	NA	NA	NA	NA
		Diarrheal Coinfection (DC)	4	4	3	0.5±0.265	0.00112 ± 0.001	1.5	Y = 1.0000 +0.0000X	0	1.716 (0.419)	-0.745 (>0.10)	-0.674 (>0.10)	-0.754 (>0.10)

	NR 1	All	11	11	6	0.891 ±0.063	0.00365 ± 0.00056	3.055	Y = 1.00 00 +0.0 000 X	0	-0.489 (0.215)	1.364 (>0.05)	1.473 (>0.05)	1.128 (> 0.10)
		Sole-Diarrheal (SD)	5	5	4	0.9±0.161	0.00382 ± 0.00083	3.2	Y = 1.00 00 +0.0 000 X	0	-0.226 (0.336)	0.763 (> 0.10)	0.791 (> 0.10)	0.763 (> 0.10)
		Non-Diarrheal (ND)	2	2	1	0	0	0	NA	NA	NA	NA	NA	NA

		Diarrheal Coinfection (DC)	4	4	3	0.833 ±0.222	0.0011± 0.0004	1	Y = 1.0000 +0.000 X	0	-0.887 (0.25)	-0.7099 (>0.10)	-0.6042 (>0.10)	-0.7099 (>0.10)
	NR2	All	11	11	5	0.855 ±0.066	0.00904 ±0.00154	7.05	Y = 1.0000 +0.000 X	0	2.953 (0.115)	1.244 (>0.10)	1.532 (<0.10)	1.673 (>0.10)
		Sole-Diarrheal (SD)	5	5	4	0.9±0.0161	0.00923 ±0.005	8.6	Y = 1.0000 +0.000 X	0	1.358 (0.428)	1.421 (>0.10)	1.514 (>0.10)	1.421 (>0.10)

		Non-Diarr heal (ND)	2	2	1	0	0	0	NA	NA	NA	NA	NA
		Diarr heal Coinf ection (DC)	4	4	3	0.833 ±0.222	0.00126 ±0.0004	1	Y = 1.0000 +0.000 X	0	-0.887 (0.25)	-0.7099 (>0.10)	-0.6042 (>0.10)	-0.7099 (>0.10)

Table 7.3: Summary table of intragenic haplotype variability of *G. lamblia* assemblage B isolates at each locus

Assemblage	**Locus**	**Population**	**N**	**S**	**H**	**Hd (S.D.)**	**Π (S.D.)**	**K**	**LD |D'|**	**Rm**	**FS (*P*)**	**FLD (*P*)**	**FLF (*P*)**	**D (*P*)**
B	ef-1α	All	20	20	9	0.826 ±0.073	0.00268 ±0.00041	3.558	Y = 0.9945 - 0.0227X	2	-1.166 (0.126)	-0.667 (> 0.10)	-0.67 (> 0.10)	-0.360 (> 0.10)
		Sole-Diarrheal (SD)	6	6	4	0.8±0.172	0.00271 ±0.00086	13.6	Y = 1.0000 + 0.0000X	0	-0.567 (0.357)	-1.16 (> 0.10)	-1.232 (> 0.10)	-1.069 (> 0.10)

		Non-Diarr heal (ND)	6	6	5	0.933 ±0.122	0.00306 ±0.00069	4.06	Y = 0.9700 + 0.0051X	1	-0.716 (0.258)	0.249 (>0.10)	0.254 (>0.10)	0.188 (>0.10)
		Diarr heal Coinf ection (DC)	8	8	6	0.893 ±0.111	0.00288 ±0.00064	3.82	Y = 0.9805 - 0.0402X	2	-1.059 (0.187)	0.139 (>0.10)	0.106 (>0.10)	-0.045 (>0.10)
	Enolase (ENOL)	All	20	20	8	0.863 ±0.049	0.00926 ±0.00079	12.38	0.9819-0.0253X	5	4.589 (0.02*)	0.124 (>0.10)	0.237 (0.02*)	1.497(>0.10)
		Sole-Diarr	6	6	3	0.733 ±0.155	0.00807 ±0.0024	10.8	Y = 1.0000 -	0	5.058	1.391 (>	1.446 (>	1.09 (>

		heal (SD)							0.0000X		(0.054)	0.10)	0.10)	0.10)
		Non-Diarrheal (ND)	6	6	6	1.000 ±0.096	0.0106± 0.0016	14.4	0.9851-0.0201X	3	-0.428 (0.395)	-0.065 (>0.10)	-0.061 (>0.10)	-0.023 (>0.10)
		Diarrheal Coinfection (DC)	8	8	7	0.964 ±0.077	0.0101± 0.0012	13.53	Y = 1.0000 +0.0000X	3	0.099 (0.354)	-0.321 (>0.10)	-0.309 (>0.10)	-0.133 (>0.10)
	NR1	All	20	20	6	0.858 ±0.032	0.0121± 0.00069	10.1	Y = 1.0193-	2	6.381	1.209 (>	1.496 (>	1.469 (>0.10)

									0.14 83X		(0.0 06)	0.1 0)	0.1 0)	
		Sole-Diarr heal (SD)	6	6	2	0.6±0. 129	0.01007 ± 0.00217	8. 4	Y = 1.00 00 +0.0 000 X	1	7.34 1 (0.0 1*)	1.6 66 (0.0 2*)	1.9 48 (0.0 2)	2.27 1 (<0. 01*)
		Non-Diarr heal (ND)	6	6	4	0.867 ±0.12 9	0.01233 ±0.0023	10 .2	1.00 92-0.13 48X	2	2.62 4 (0.2 32)	0.3 77 (>0 .10)	0.4 09 (>0 .10)	0.36 7 (>0. 10)
		Diarr heal Coinf ection (DC)	8	8	5	0.857 ±0.10 8	0.01233 ±0.0019 5	10 .2 8	Y = 1.01 03 - 0.06 64X	1	2.46 2 (0.1 94)	0.3 9 (>0 .10)	0.4 85 (>0 .10)	0.58 4 (>0. 10)

	NR 2	All	20	20	8	0.837 ±0.059	0.01862 ±0.00247	14.8	Y = 0.9876-0.0669X	5	5.638 (0.01*)	1.571 (0.02*)	1.592 (<0.10)	0.885 (>0.10)
		Sole-Diarrheal (SD)	6	6	2	0.6±0.129	0.01887 ±0.00406	15	Y = 1.0000 +0.000X	0	9.649 (0.002*)	1.71 1 (0.02*)	2.011 (0.02)	2.33 (<0.01*)
		Non-Diarrheal (ND)	6	6	6	1.000 ±0.096	0.01635 ±0.00303	13	Y = 1.0000 - 0.0000X	0	- 0.57 (0.361)	- 0.134 (>0.10)	- 0.131 (>0.10)	- 0.066 (>0.10)

		Diarrheal Coinfection (DC)	8	8	5	0.857 ±0.108	0.01837 ±0.00443	14.6	Y = 0.9855 +0.059X	3	3.393 (0.12)	-0.573 (>0.10)	-0.627 (>0.10)	-0.526 (>0.10)

N = number of samples, *S* = number of sequences, *H* = haplotypes, Hd = haplotype diversity, Π = nucleotide diversity, *K* = average, LD= linkage disequilibrium, R_m= Number of recombination events, FS = Fu's Fs, FLD= Fu Li's D, FLF= Fu Li's F, D = Tajima's D, S.D. = standard deviation, *P* = *P* value

7.3.5. Linkage disequilibrium (LD) and recombination analyses

7.3.5.1. Assemblage A

Intragenic linkage disequilibrium (LD) and potential recombination events were evaluated for each target locus. In Assemblage A, all four loci exhibited complete intragenic LD (|D′| Y = 1.0000 ± 0.0000X), where Y represents the LD value and X denotes the nucleotide distance in kilobases. This complete intragenic LD across all four loci suggests a random distribution of alleles for these genes within the *G. lamblia* Assemblage A population. Analysis of intragenic recombination detected no recombination events at any of these loci in this population (Table 7.2).

7.3.5.2. Assemblage B

Assessment of assemblage B intragenic linkage disequilibrium (LD) and potential recombination events for each target locus revealed incomplete intragenic LD values across all loci, suggesting a non-random distribution of alleles within the population. Interestingly, the sole diarrheal group exhibited complete LD values ($|D'| = 1.0000 - 0.0000X$) for all loci, contrasting with the other groups. The non-diarrheal group showed incomplete LD values at ef-1α, Enolase, and NR1 loci, while the diarrheal coinfection group displayed incomplete values at ef-1α, NR1, and NR2 loci. Intragenic recombination analysis identified potential events (Rm) at all loci, albeit with varying frequencies; Enolase and NR2 showed the highest number of events (5 each), while ef-1α and NR1 had the lowest (2 each). These findings highlight the complex genetic structure and recombination patterns among the different groups and loci studied in assemblage B (Table 7.3).

7.3.6. Population structure

7.3.6.1. Assemblage A

The population genetic analysis of assemblage A revealed complex evolutionary dynamics across different loci and groups. Tajima's D, Fu and Li's D, and Fu and Li's F statistics yielded positive values when combining all three groups across all loci. However, a more nuanced picture emerged when examining individual groups and loci. The sole diarrhea group exhibited negative values for the ef-1α locus, suggesting recent population expansion, while maintaining positive values for other loci, potentially indicating a population bottleneck or balancing selection. The diarrheal coinfection group displayed positive values for all three statistics at the ef-1α locus but negative values for the remaining loci, highlighting differential evolutionary trajectories among the tested loci. Unfortunately, neutrality tests could not be performed for the non-diarrheal group due to

insufficient sample size (n=2). These contrasting results underscore the heterogeneous evolutionary pressures acting on different loci within and across groups (Table 7.2).

7.3.6.2. Assemblage B

Population genetic analysis of assemblage B revealed following results. Neutrality tests, including Tajima's D, Fu and Li's D, and Fu and Li's F statistics, yielded negative values for the ef-1α locus and positive values for all other loci when combining data from all three groups. The sole diarrhea group exhibited a distinct pattern, with negative values at the ef-1α locus and positive values for the remaining loci. Interestingly, the non-diarrheal and diarrheal coinfection groups demonstrated similar trends in neutrality tests, both showing positive values at the ef-1α and NR1 loci, while displaying negative values at the Enolase and NR2 loci. This parallel pattern suggests that these two groups may have undergone similar evolutionary trajectories across the examined loci. In contrast, the sole diarrheal group exhibited distinct evolutionary dynamics, indicating differential selective pressures or demographic events affecting this group (Table 7.3). Including various groups in the haplotype network did not lead to any noticeable impact on the grouping of isolates (Fig 7.5-7.8).

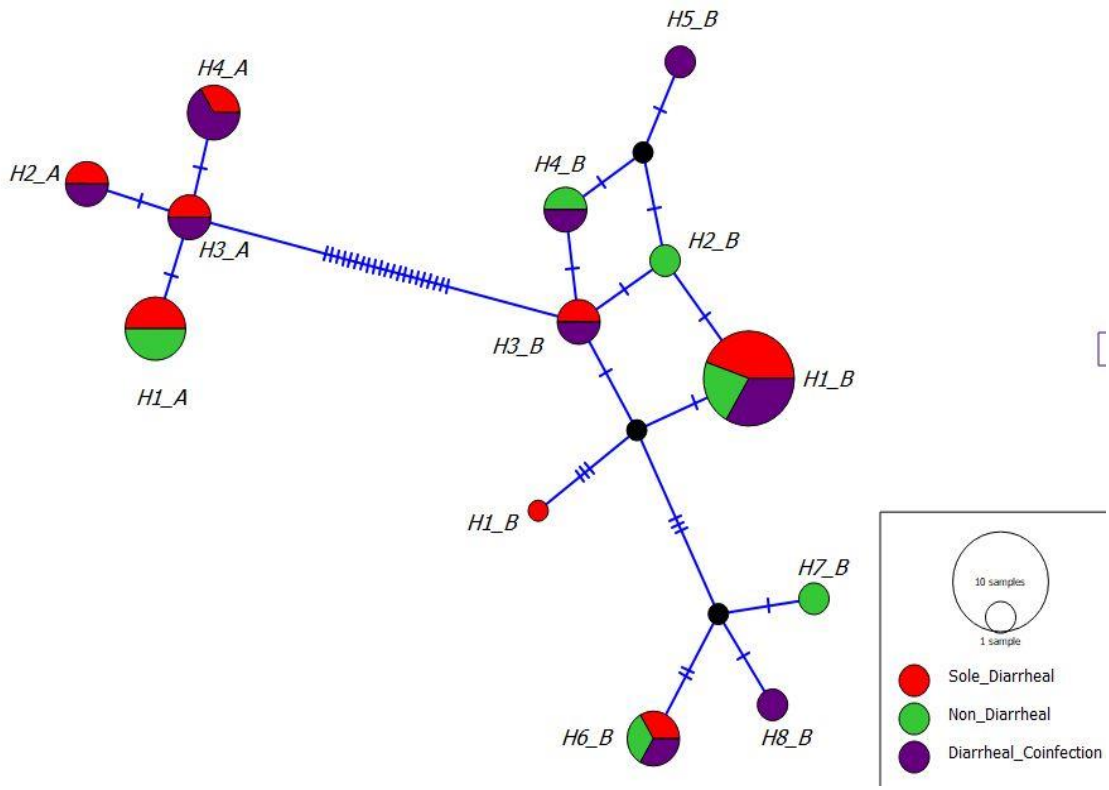


Fig 7.5: The TCS haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the ef-1α gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

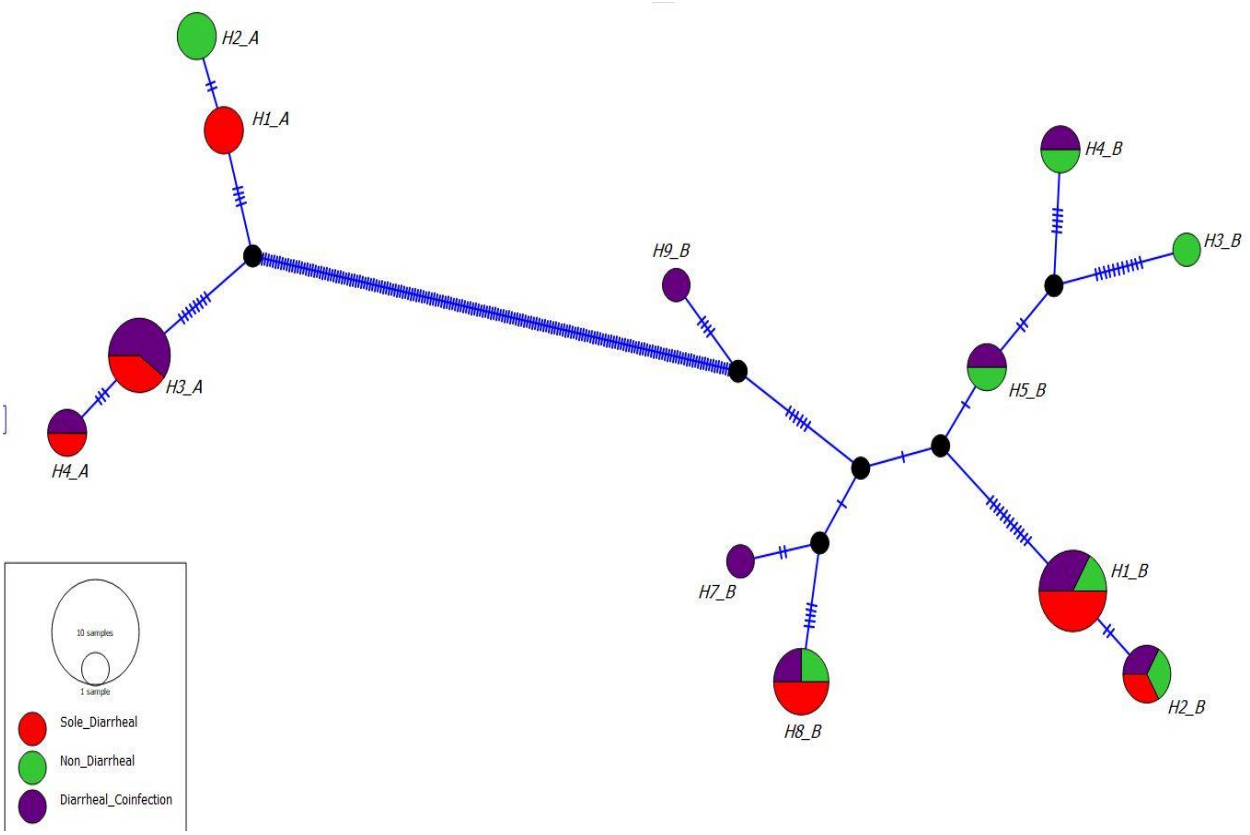


Fig 7.6: The TCS haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the Enolase gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

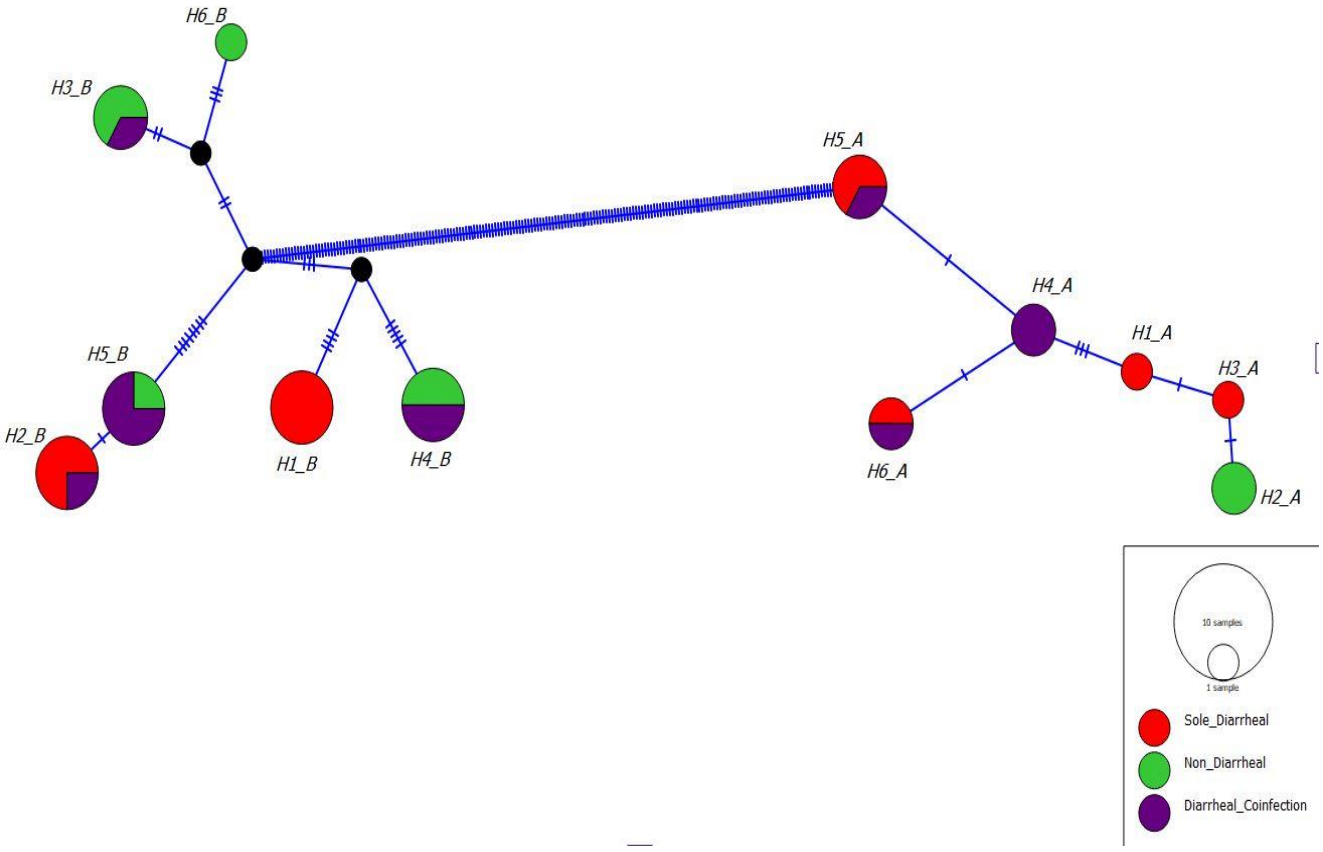


Fig 7.7: The TCS haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the NR1 gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

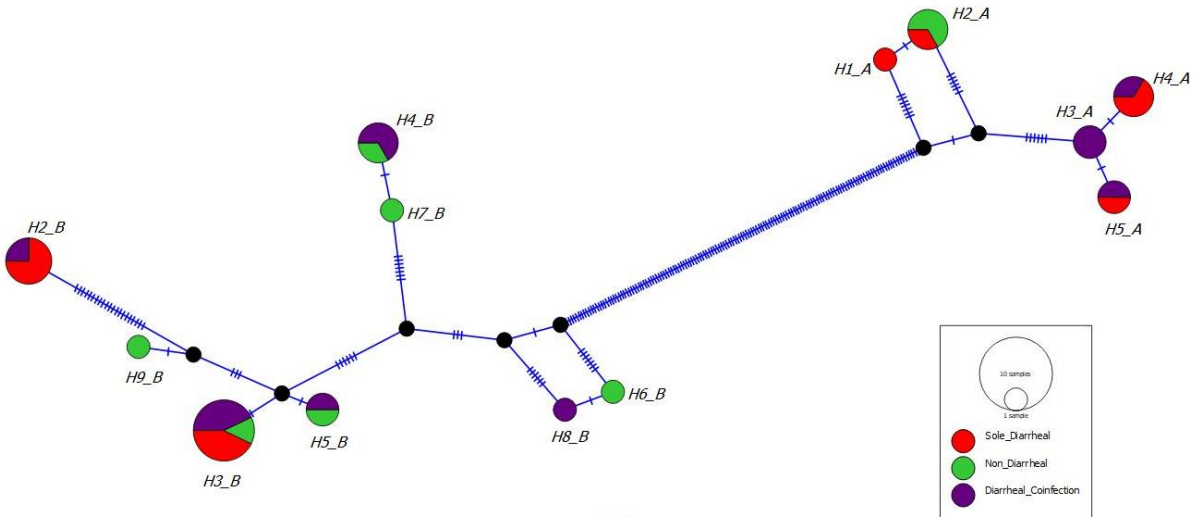


Fig 7.8: The TCS haplotype network analysis of *Giardia lamblia* assemblages A and B sequences, based on the NR2 gene, is presented. The size of each circle in the network is proportional to the frequency of the corresponding haplotype (n), while the black circles represent extinct or unsampled haplotypes. The hatch marks on the lines connecting the haplotypes indicate the mutational steps between them. The colors of the haplotypes represent their geographic locations, as depicted in the legend on the right corner of the figure.

7.4. Discussion

In this study, we examined the genetic diversity of four full-length genes in local isolates of *Giardia lamblia*: elongation factor 1-alpha (ef-1α), Enolase and two variants of the Nitroreductase gene (NR1 and NR2). These genes are regarded as potential virulence factors of the pathogen. The molecular mechanisms underlying the pathogenesis of the intestinal protozoan parasite *Giardia* still remain obscure. However, excretory-secretory products are hypothesized to play a crucial role in host-parasite interactions (Skarin et al., 2011). In our study, we successfully amplified and sequenced two genes encoding secretory proteins, namely Enolase and ef-1α, from multiple local isolates representing both major assemblages of *Giardia*. Elongation factor 1-alpha (ef-1α) exhibits dynamic subcellular localization in *Giardia* trophozoites. Under normal conditions, it is predominantly found in the nuclear region. However, during host-cell interactions, ef-1α

undergoes translocation to the cytoplasm, suggesting a significant role in the infection process (Skarin et al., 2011). ef-1α and similar proteins have been implicated as virulence factors in various pathogenic organisms, including bacteria and other protozoan parasites such as *Leishmania* species (Nandan et al., 2002). This conservation across diverse pathogens underscores the potential importance of ef-1α in *Giardia* pathogenesis. The ef-1α locus exhibits a pattern of low nucleotide diversity coupled with high haplotype diversity, suggesting the presence of multiple closely related haplotypes within the population. This pattern is indicative of a relatively conserved genomic region. Furthermore, the locus demonstrates a paucity of non-synonymous single nucleotide variants (nsSNVs). Neutrality tests for this locus yielded a negative value, signifying an excess of diverse haplotypes compared to expectations under neutrality. This finding is typically associated with recent population expansion. A negative result, indicates an overrepresentation of rare nucleotide variants relative to the expected frequency under a neutral model of evolution (Ghosal et al., 2023). These observations collectively suggest a recent demographic expansion of a particular haplotype within the population. This expansion may be driven by potentially advantageous traits conferred by the haplotype, which could enhance fitness across diverse environmental conditions.

Enolase is a multifunctional protein expressed by various pathogens. It plays a crucial role in early host-pathogen interactions by activating plasminogen, which in turn promotes the proteolysis of extracellular matrix components. In the context of *G. lamblia*, a secreted form of enolase has been identified in the secretome. This form is released when trophozoites interact with epithelial cells. Despite this discovery, the specific functions and mechanisms of enolase in *G. lamblia* host-pathogen interactions remain largely uncharacterized (Barroeta-Echegaray et al., 2022). Our study of the locus revealed moderate nucleotide diversity coupled with high haplotype diversity. The

assemblage A population exhibited a negative value in neutrality tests. This suggests recent positive selection or population growth of certain haplotype. Conversely, the assemblage B population demonstrated a positive value in neutrality tests. This result implies the presence of relatively low levels of both low- and high-frequency polymorphisms within the population, consistent with balancing selection. Balancing selection actively maintains multiple alleles at frequencies higher than expected under neutral evolution, potentially contributing to increased average population fitness (Ghosal et al., 2023). Notably, the assemblage B population also exhibited the highest number of recombination events at the enolase locus. This observation aligns with expectations under balancing selection, as recombination can generate and maintain diverse haplotypes. These findings suggest differential selective pressures acting on the enolase locus between assemblages A and B of *G. lamblia*. While assemblage A shows signs of directional selection or population expansion, assemblage B exhibits characteristics consistent with balancing selection, potentially maintaining functional diversity at this locus.

Metronidazole (MTZ) activation in *Giardia* involves multiple enzymes, including NR1, PFOR1 (and potentially PFOR2), and thioredoxin reductase, while NR2 is associated with its inactivation (Saghaug et al., 2023). The development of MTZ resistance in *Giardia* is likely pleiotropic, encompassing various mechanisms such as downregulation of MTZ-activating enzymes, upregulation of MTZ-inactivating enzymes, genetic variations, and post-transcriptional modifications (Ansell et al., 2015, 2017; Leitsch, 2019; Müller et al., 2018). This complex resistance profile may involve multiple metabolic systems that protect against reactive toxic metabolites. Different *Giardia* strains may potentially evolve distinct resistance strategies through combinations of these mechanisms, highlighting the multifaceted nature of MTZ resistance in this parasite. Of all these enzymes, we have been able to sequence and check for variations in NR1 and

NR2. Both these loci had the highest number of sSNVs and nsSNVs. Both assemblages exhibited high nucleotide and haplotype diversity, coupled with positive values across all neutrality tests, suggesting the presence of balancing selection at these two loci. This evolutionary mechanism maintains multiple alleles at frequencies exceeding those expected under neutral evolution. This genetic landscape implies that, under the selective pressure of metronidazole (MTZ) in the environment, the parasite retains genetic variations that potentially alter the native protein structures, rendering them inactive. Consequently, this may lead to either failed MTZ activation or its inactivation, resulting in drug-resistant isolates (Saghaug et al., 2023). This scenario underscores the adaptive capacity of the parasite in response to drug-induced selective pressures, highlighting the complex interplay between genetic variation and drug resistance mechanisms. However, further investigation is warranted with a larger sample size and including other loci to validate these findings.

The substantial genetic diversity observed in virulence-associated genes within this *G. lamblia* population suggests a significant potential for genomic plasticity. Such variation may influence metronidazole (MTZ) susceptibility profiles and enhance the parasite's overall adaptive capacity across diverse environmental conditions. This genetic heterogeneity could facilitate rapid evolutionary responses to selective pressures, like drug exposure and host immune responses. The observed polymorphisms may contribute to phenotypic variations in virulence factors, potentially impacting host-parasite interactions and treatment outcomes.

7.5. Conclusion

The genetic analysis of *Giardia* assemblages reveals significant variation in the metronidazole (MTZ)-metabolizing genes NR1 and NR2, suggesting a high potential for genetic alterations that could impact MTZ susceptibility and resistance profiles. This extensive polymorphism in

nitroreductase genes underscores their pivotal role in the parasite's adaptive response under MTZ selective pressure. In contrast, the genes encoding ef-1α and enolase exhibit comparatively lower genetic variation. This differential pattern of genetic diversity across loci indicates the presence of distinct selective pressures acting on various components of the parasite's genome. The relative conservation of ef-1α and enolase suggests these genes may be under purifying selection, potentially due to their crucial roles in basic cellular functions and host-parasite interactions. The observed differences in the extent of genetic variation across these loci points to a multifactorial and complex mechanism of pathogenesis in *Giardia*. This complexity is likely driven by the interplay between maintaining essential cellular functions and adapting to environmental pressures, particularly drug exposure.

Chapter 8

SUMMARY

Enteric parasitic infections continue to pose significant public health challenges, especially in regions with environmental conditions conducive to pathogen transmission. This study aimed to investigate the prevalence, genetic diversity, and clinical implications of enteric protozoan parasites, with a particular focus on *Giardia lamblia*, among both diarrheal patients and asymptomatic individuals in eastern India. Notably, the study also identified the presence of relatively novel enteric parasites, such as *Entamoeba moshkovskii*, *Cryptosporidium viatorum*, and *Cyclospora cayetanensis*, which have not been previously reported in this region but exhibited substantial prevalence rates.

The overall prevalence of intestinal parasitic infections among diarrheal patients was found to be quite high in the region, with *G. lamblia* emerged as the most prevalent protozoan. A clear seasonal trend was observed for all the mentioned parasites, with higher infection rates during humid rainy seasons compared to dry winter months. The community-based survey of asymptomatic individuals revealed a higher prevalence of *G. lamblia* compared to symptomatic cases. Asymptomatic carriage was particularly prevalent in the 2-20 years age group, suggesting this population may serve as a reservoir for infection. Consistent with findings from symptomatic co-infections, assemblage B remained the dominant genotype in asymptomatic cases. This is the first ever report of prevalence of *G. lamblia* in asymptomatic population in eastern India. Genotyping study revealed two distinct assemblages of *Giardia lamblia*- assemblage A and assemblage B. Assemblage B demonstrated a significantly higher prevalence, occurring twice as frequently as assemblage A. Within assemblage A, subtype AII was more common than AI. Notably, a potential correlation between assemblage A and symptomatic giardiasis was observed. The high prevalence of *G. lamblia*, in both symptomatic and asymptomatic populations, underscores its significant public health importance in the region. The higher prevalence of assemblage B, in both

symptomatic and asymptomatic cases, indicates its successful adaptation to human hosts in this geographical area. No indigenous rapid detection method is currently available in India for efficiently identifying and genotyping of human isolates of *G. lamblia*. We were able to develop a multiplex PCR-RFLP method with excellent diagnostic performance. The method demonstrated a high sensitivity (94.2%), specificity (100%), and accuracy (97.1%) for giardiasis diagnosis. Our innovative approach enabled simultaneous identification and differentiation of assemblages A and B, as well as subtyping of AI, AII, BIII, and BIV in a rapid and cost-effective manner. Genetic analysis of virulence genes and MTZ metabolic pathway genes revealed higher allelic diversity and a greater number of non-synonymous single nucleotide variations (SNVs) in assemblage B compared to assemblage A. The observed genetic diversity in virulence and MTZ metabolism genes, particularly in assemblage B, raises concerns about the potential for drug resistance and parasite adaptability. The higher number of non-synonymous SNVs in assemblage B suggests that these genetic variations may confer functional changes that enhance the parasite's survival and virulence. This finding underscores the need for continued surveillance of drug-resistant isolates of *G. lamblia* in the population and the development of alternative treatment strategies. The results from the study can be of importance to control giardiasis, especially in vulnerable populations such as children and asymptomatic carriers in eastern India.

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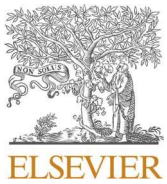
Experimental Parasitology 262 (2024) 108773



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Andrographolide induced cytotoxicity and cell cycle arrest in *Giardia* trophozoites

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ABSTRACT

Giardiasis is a prevalent parasitic diarrheal disease caused by *Giardia lamblia*, affecting people worldwide. Recently, the availability of several drugs for its treatment has highlighted issues such as multidrug resistance, limited effectiveness and undesirable side effects. Therefore, it is necessary to develop alternative new drugs and treatment strategies that can enhance therapeutic outcomes and effectively treat giardiasis. Natural compounds show promise in the search for more potent anti-giardial agents. Our investigation focused on the effect of Andrographolide (ADG), an active compound of the *Andrographis paniculata* plant, on *Giardia lamblia*, assessing trophozoite growth, morphological changes, cell cycle arrest, DNA damage and inhibition of gene expression associated with pathogenic factors. ADG demonstrated anti-*Giardia* activity almost equivalent to the reference drug metronidazole, with an IC_{50} value of 4.99 μM after 24 h of incubation. In cytotoxicity assessments and morphological examinations, it showed significant alterations in trophozoite shape and size and effectively hindered the adhesion of trophozoites. It also caused excessive ROS generation, DNA damage, cell cycle arrest and inhibited the gene expression related to pathogenesis. Our findings have revealed the anti-giardial efficacy of ADG, suggesting its potential as an agent against *Giardia* infections. This could offer a natural and low-risk treatment option for giardiasis, reducing the risk of side effects and drug resistance.

1. Introduction

Giardia lamblia is one of the most frequent protozoan parasites that cause giardiasis worldwide. According to WHO reports annually 280 million people have been infected globally (Carter et al., 2018). Giardiasis is becoming an increasingly prevalent issue, particularly in developing country, as it continues to emerge and spread within human populations. In developed countries, the prevalence rate of giardiasis is between 2 and 5%, while in developing countries ranges from 20 to 30% (Lalle and Hanevik, 2018). The disease is typically transmitted indirectly through the consumption of food or water that has been contaminated with cysts or through person-to-person contact, especially among individuals living in unhygienic conditions. Although giardiasis can affect individuals of all ages and display symptoms, it tends to affect children more frequently than adults (Belkessa et al., 2021). Giardiasis can either be asymptomatic or result in symptoms such as diarrhoea, vomiting, flatulence, anorexia, and crampy abdominal pain during its acute phase (Escobedo et al., 2018). In addition to gastrointestinal symptoms, it may also lead to extra-intestinal manifestations such as fever, maculopapular rash, pulmonary infiltrate, lymphadenopathy, polyarthritis, urticaria and growth retardation. Metronidazole is considered the first-line treatment for giardiasis, but alternative medications like albendazole, furazolidone and tinidazole are also used (Beer et al., 2017). Adverse effects of common drugs results headaches, dizziness, metallic taste, low efficacy and treatment failure have been reported as potential issues that cannot be overlooked (Starrs and Yenigun, 2021). The development of resistance to chemotherapeutic drugs among parasites underscores the critical importance of exploring alternative therapeutic approaches. One such approach involves investigating natural plant products as potential novel anti-parasitic agents (Anthony et al., 2005).

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

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Abstract

Entamoeba moshkovskii, according to recent studies, appears to exert a more significant impact on diarrhoeal infections than previously believed. The efficient identification and genetic characterization of *E. moshkovskii* isolates from endemic areas worldwide are crucial for understanding the impact of parasite genomes on amoebic infections. In this study, we employed a multilocus sequence typing system to characterize *E. moshkovskii* isolates, with the aim of assessing the role of genetic variation in the pathogenic potential of *E. moshkovskii*. We incorporated 3 potential genetic markers: KERP1, a protein rich in lysine and glutamic acid; amoebapore C (apc) and chitinase. Sequencing was attempted for all target loci in 68 positive *E. moshkovskii* samples, and successfully sequenced a total of 33 samples for all 3 loci. The analysis revealed 17 distinct genotypes, labelled M1–M17, across the tested samples when combining all loci. Notably, genotype M1 demonstrated a statistically significant association with diarrhoeal incidence within *E. moshkovskii* infection ($P = 0.0394$). This suggests that M1 may represent a pathogenic strain with the highest potential for causing diarrhoeal symptoms. Additionally, we have identified a few single-nucleotide polymorphisms in the studied loci that can be utilized as genetic markers for recognizing the most potentially pathogenic *E. moshkovskii* isolates. In our genetic diversity study, the apc locus demonstrated the highest H_d value and π value, indicating its pivotal role in reflecting the evolutionary history and adaptation of the *E. moshkovskii* population. Furthermore, analyses of linkage disequilibrium and recombination within the *E. moshkovskii* population suggested that the apc locus could play a crucial role in determining the virulence of *E. moshkovskii*.

Introduction

Amoebic infection is a complex issue as several species are morphologically indistinguishable from each other, including *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba bangladeshi* and *Entamoeba 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. The cysts of a non-pathogenic amoeba, *Entamoeba hartmanni*, can also be mistaken with the pathogenic *E. histolytica* under a microscope (Burrows, 1959) adds an uncertainty. While *E. histolytica* is known to cause pathogenicity in amoebic infections, the actual prevalence of this species is likely overestimated due to these morphological overlaps. Recent research has indicated that *E. moshkovskii* might have a more significant impact on human infections than previously believed. This species has been detected in multiple countries, including the United States, Italy, Iran, Turkey, Indonesia, Colombia, Bangladesh, India, Kenya, Australia, Malaysia, Tanzania, Tunisia and Brazil (Ali *et al.*, 2003; 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). In addition, *E. moshkovskii* has been identified in farm animals such as pigs, showcasing its potential for zoonotic transmission (Sardar *et al.*, 2022). Moreover, it has been documented in non-human primates as well (Levecke *et al.*, 2010). A study conducted in eastern India by Sardar *et al.* (2023*a*, 2023*b*) also revealed that *E. moshkovskii* is one of the causative agents of diarrhoeal incidents in humans. The research found that many patients suffering from diarrhoea, infected with *E. moshkovskii*, tested negative for other common enteric pathogens such as bacteria and viruses (Sardar *et al.*, 2023*a*, 2023*b*). These findings, combined with various studies conducted in different regions, suggest the potential pathogenicity of *E. moshkovskii* in humans. Therefore, diagnosing diarrhoeal patients should include consideration of *E. moshkovskii* as a potential pathogen to ensure accurate identification of the causative agent. Neglecting this can result in undetermined cases of diarrhoeal illness, leading to improper drug treatments for patients.

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Multi-locus sequence analysis reveals phylogenetically segregated *Entamoeba histolytica* population.

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ARTICLE INFO

Keywords:
Amoebiasis
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Disease outcome
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ABSTRACT

Amoebiasis, caused by the enteric parasite, *Entamoeba histolytica*, is one of the major food- and water-borne parasitic diseases in developing countries with improper sanitation and poor hygiene. Infection with *E. histolytica* has diverse disease outcomes, which are determined by the genetic diversity of the infecting strains. Comparative genetic analysis of infecting *E. histolytica* strains associated with differential disease outcomes from different geographical regions of the world is important to identify the specific genetic patterns of the pathogen that trigger certain disease outcomes of Amoebiasis. The strategy is able to elucidate the genealogical relation and population structure of infecting *E. histolytica* strains from different geographical regions. In the present study, we have performed a comparative genetic analysis of circulating *E. histolytica* strains identified from different parts of the world, including our study region, based on five tRNA-linked short tandem repeat (STR) loci (i.e., *D-A*, *N—K2*, *R-R*, S^{TGA}*-D* and *A-L*) and evaluated their potential associations with differential disease outcomes of Amoebiasis. A number of regional-specific, emerging haplotypes of *E. histolytica*, significantly associated with specific disease outcomes have been identified. Haplotypes, which have a significant positive association with asymptomatic and amoebic liver abscess outcomes, showed a significant negative association with diarrheal outcome, or vice versa. Comparative multi-locus analysis revealed that *E. histolytica* isolates from our study region are phylogenetically segregated from the isolates of other geographical regions. This study provides a crucial overview of the population structure and emerging pattern of the enteric parasite, *E. histolytica*.

1. Introduction

Entamoeba histolytica, an enteric protozoan parasite causes Amoebiasis in humans [1]. This is the second leading cause of death due to parasite infection after malaria [2]. Countries with highest prevalence of *E. histolytica* infection include Mexico, India, East and South Africa and regions of Central and South America [3]. Estimation of the worldwide burden of Amoebiasis suggested that approximately 500 million people were infected by this parasite and only 10% of those individuals' developed symptoms of invasive disease [4,5]. Infection is usually acquired through the ingestion of cysts of *Entamoeba* sp. in sewage contaminated food and water. Cysts are infective form of parasite excreted through feces from asymptomatic cyst passers or patients with symptomatic amoebic infection. Excystation takes place in the small intestine, produce motile trophozoites that colonize the large intestine. Under the circumstances, trophozoites can destroy the intestinal mucosa and invade deeper into intestinal structure, causing the known Bottle Neck Ulcer. On rare occasions, they are exported by mesenteric and portal circulatory system in the liver and cause amoebic liver abscess like rare but fatal outcomes [4]. The factors that explain why *E. histolytica* infections in humans become sporadically symptomatic are still controversial. Many reports suggested that such variations in

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Short tandem repeat (STR) based sequence typing of *Entamoeba histolytica* identifies S^{TGA}-*D* locus as a genetic marker, associated with disease outcomes.

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ARTICLE INFO

Keywords:
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tRNA linked STR loci
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ABSTRACT

Amoebiasis, caused by the enteric parasite *Entamoeba histolytica* has differential disease outcomes. The association of parasite genotypes with outcomes of amoebic infection is still a paradox and requires to be explored. The genetic information of infecting strains from endemic settings of different geographical regions is essential to evaluate the relation. Comparative genetics of *E. histolytica* clinical isolates from different disease outcomes have been explored based on two tRNA-linked STR loci (S^{TGA}-*D* and *A*-*L*). All of the repeat patterns in the *A*-*L* locus were newly identified and unique to Indian isolates. The majority of newly identified repeat patterns in S^{TGA}-*D* locus have outcome-specific distributions, predicting the emergence of disease-specific mutations in this target locus. Statistical analysis further reinforces this observation, as identified repeat patterns only from S^{TGA}-*D* but not *A*-*L* locus were significantly associated with disease outcomes. Phylogenetic analysis indicates independent segregation and divergence of tRNA-linked STR arrays for each STR locus.

1. Introduction

Amoebiasis, a major enteric disease affecting human around the world, is caused by a protozoan parasite *Entamoeba histolytica*. It is one of the major parasitic diseases after Malaria and is responsible for an estimated 50 million human infections around the world with about 40–100 thousand deaths per year [1–4]. This parasitic infection has diverse clinical manifestations. Majority of infections remain asymptomatic while some lead to intestinal complications like diarrhea and dysentery. Moreover, *E. histolytica* infection can sporadically give rise to fatal extra-intestinal manifestations such as amoebic liver abscesses [1,5]. Potential factors for these differential disease outcomes are highly debated; however, few previous studies indicate that host genetics and parasite genotypes could play crucial roles [6,7]. Genetic characterization of the clinical isolates from different geographic regions throughout the world therefore could elucidate the association between the parasite genotypes and outcomes of amoebic infection. Single locus-based genotyping method (exploits single polymorphic markers such as Serine rich *Entamoeba histolytica* protein (SREHP), Chitinase (CHI) gene, etc.) has limited application due to low resolution [8–11]. In contrast, multilocus sequence typing based on repetitive,non-coding tRNA-linked short tandem repeat (STR) loci has emerged as a high-resolution, well-accepted method to determine the population structure of *E. histolytica* and to evaluate the potential association between parasite genotypes and outcomes of amoebic infection [12–15]. The tRNA genes are recognized as “hotspots” for recombination and mutation due to their unique telomeric location and genetic organization, making them loci of choice for evolutionary study of *E. histolytica* [16].

In the present study, genotyping of *E. histolytica* clinical isolates obtained from individuals with different disease outcomes (i.e., from

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Original Article

Phylogenetic analyses of *Chilomastix* and *Retortamonas* species using *in vitro* excysted flagellates

Análise filogenética das espécies de *Chilomastix* e *Retortamonas* utilizando flagelados excistados *in vitro*

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Abstract

In vitro excystation of cysts of microscopically identified *Chilomastix mesnili* and *Retortamonas* sp. isolated from Japanese macaques and *Retortamonas* sp. isolated from small Indian mongooses could be induced using an established protocol for *Giardia intestinalis* and subsequently by culturing with H_2S-rich Robinson's medium supplemented with *Desulfovibrio desulfuricans*. Excystation usually began 2 h after incubation in Robinson's medium. DNA was isolated from excysted flagellates after 4 h of incubation or from cultured excysted flagellates. Phylogenetic analysis based on their 18S rRNA genes revealed that two isolates of *C. mesnili* from Japanese macaques belonged to the same cluster as a *C. mesnili* isolate from humans, whereas a mammalian *Retortamonas* sp. isolate from a small Indian mongoose belonged to the same cluster as that of an amphibian *Retortamonas* spp. isolate from a 'poison arrow frog' [sequence identity to AF439347 (94.9%)]. These results suggest that the sequence homology of the 18S rRNA gene of the two *C. mesnili* isolates from Japanese macaques was similar to that of humans, in addition to the morphological similarity, and *Retortamonas* sp. infection of the amphibian type in the small Indian mongoose highlighted the possibility of the effect of host feeding habitats.

Keywords: Excystation, *Chilomastix mesnili*, *Retortamonas* spp., *Macaca fusucata*, *Urva auropunctata*, phylogenetic analysis.

Resumo

Excistação *in vitro* de cistos de *Chilomastix mesnili* e *Retortamonas* sp. identificados microscopicamente, isolados de macacos japoneses e *Retortamonas* sp. isolados de um pequeno mangusto indiano podem ser induzidos, usando-se um protocolo estabelecido para *Giardia intestinalis* e, subsequentemente, por cultura com meio de Robinson rico em H_2S, suplementado com *Desulfovibrio desulfuricans*. A excistação geralmente começou 2 h após a incubação em meio de Robinson. O DNA foi isolado de flagelados excistados, após 4 h de incubação, ou de flagelados excistados cultivados. A análise filogenética baseada em seus genes 18S rRNA revelou que dois isolados de *C. mesnili* de macacos japoneses pertenciam ao mesmo grupo que um isolado de *C. mesnili* de um humano, enquanto um isolado de *Retortamonas* sp. de um mamífero, o pequeno mangusto indiano, pertencia ao mesmo agrupamento do isolado de *Retortamonas* spp. do anfíbio, 'sapo ponta-de-flecha' [identidade sequencial para AF439347 (94,9%)]. Esses resultados sugeriram que a homologia sequencial do gene 18S rRNA, dos dois isolados de *C. mesnili* de macacos japoneses era semelhante a de um humano, além da semelhança morfológica, e a infecção de *Retortamonas* sp. do tipo anfíbio ao pequeno mangusto indiano destacou a possibilidade de ser afetado pelo habitat alimentar de seu hospedeiro.

Palavras-chave: Excistação, *Chilomastix mesnili*, *Retortamonas* spp., *Macaca fusucata*, *Urva auropunctata*, análise filogenética.

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RESEARCH

Genotyping and epidemiological distribution of diarrhea-causing isolates of *Giardia duodenalis* in southeastern part of West Bengal, India

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Abstract

The prevalence and genetic diversity of the protozoan pathogen *Giardia duodenalis* have been extensively studied worldwide. There is currently a lack of data regarding the genetic variability of the organism in eastern India. Understanding the circulating genotypes and associated risk factors is crucial for effective planning and implementing control measures. Therefore, the objective of the study was to conduct an epidemiological study to determine the prevalence and identify the various genotypes present. This survey adds to our knowledge on the occurrence and distribution of *Giardia* genotypes in the studied region. The overall prevalence was found to be 6.8%. This parasitic infection was significantly associated with two age groups, i.e., >0–5 years and >5–12 years. Using a multilocus genotyping method, we genotyped 52 human *Giardia* isolates that were obtained from diarrheal patients. Two distinct assemblages were found in the population—30.8% belonged to assemblage A; 63.5% belonged to assemblage B, prevalent in the population; and 5.7% belonged to a combined assemblage A+B. Sub-assemblage AII was found in 17.3% of the cases, followed by sub-assemblage AI (13.5%). High levels of genetic diversity were found within the population of assemblage B undergoing balancing selection. Overall, the high prevalence of the parasite observed, particularly among children, raises a major concern and necessitates implementation of robust control measures. Furthermore, we report the presence of numerous unique genotypes, circulating in this limited geographical boundary, which can be useful dataset for future studies.

Keywords *Giardia* · MLG · SNP · Balancing selection · West Bengal · India

Introduction

Enteric parasites contribute significantly to the burden of diarrheal diseases worldwide. While parasitic diseases run rife in developing countries, sporadic cases and outbreaks are also reported in developed countries (Fletcher et al. 2012). *Giardia duodenalis* (formerly known as *G. lamblia* or *G. intestinalis*) is a non-invasive intestinal protozoan which is commonly associated with diarrheal illnesses (namely giardiasis) in humans, livestock, and wild animals (Takumi et al. 2012). About 280 million cases are reported annually (Lane and Lloyd 2002; Squire and Ryan 2017), with more than 2.5 million annual diarrhea cases coming from developing countries (Thompson 2000). Infection is initiated in the host when the ingested cysts excyst into trophozoites in the duodenum (proximal part of the small intestine) after passing through the acidic environment of the stomach. The trophozoites replicate and remain adhered on the mucosal surface of the intestine, where it causes malabsorption resulting in diarrheal symptoms. Exposure to biliary fluid causes trophozoites to turn into the resistant "cyst" form in the jejunum, which are then shed with the feces to the environment, thereby completing the transmission cycle via the fecal-oral route (Adam 2001).

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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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Functional characterization of phospholipase B enzyme from *Giardia lamblia*

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ARTICLE INFO

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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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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prevalence and molecular characterization of *Entamoeba moshkovskii* in diarrheal patients from Eastern India

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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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Case Report

First case report of Cyclosporiasis from eastern India: Incidence of *Cyclospora cayetanensis* in a patient with unusual diarrheal symptoms

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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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▣ BRIEF COMMUNICATION

Molecular Identification of *Cryptosporidium viatorum* Infection in a Patient Suffering from Unusual Cryptosporidiosis in West Bengal, India

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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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Multilocus sequence typing (MLST) of *Entamoeba histolytica* identifies *kerp2* as a genetic marker associated with disease outcomes

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ABSTRACT

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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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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ABSTRACT

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.

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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ORIGINAL RESEARCH PAPER

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PREVALENCE MAPPING OF SOIL TRANSMITTED HELMINTHS IN NORTH EASTERN STATES OF INDIA – FIRST REPORT.

Parasitology

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KEYWORDS

INTRODUCTION

Soil transmitted helminthiases (STH) is a major health problem in tropical and subtropical regions of the world, with 1.5 billion people affected worldwide. [1] There are four intestinal nematode species, namely roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*), and the hookworms (*Necator americanus* and *Ancylostoma duodenale*), that fall under the group of soil transmitted helminths. [2] Sub-Saharan Africa, East Asia, China, India and South America, contribute highest number of STH infections in the world. WHO estimates show that > 1.2 billion people are affected with ascariasis. Trichuriasis and hookworm infection have been estimated to be ~700–800 million each, globally. [3] The warm and moist climate of tropics and subtropics favours hatching and embryonation of the eggs. Soil contaminated with the egg or larvae leads to widespread infection of all the STH. [2] Besides, inadequate sanitation, open defecation, poor hygiene practices and socio-economic status in those countries contribute to high disease burden among population. STH infections causes nutritional deficiency, anaemia, growth retardation and impaired cognitive development which may result in absenteeism and disability adjusted life years (DALYs) loss. [2,4] School age children (SAC) are very much prone to be affected and normally harbour worm burdens greater than adults. According to WHO estimation, over 267 million preschool-age children and over 568 million school-age children are at risk of STH infection worldwide, of which 241 million are from India alone. [1, 4] WHO recommended annual or biannual school-based deworming can be an effective strategy to control infection in SAC. [4] Most of the studies on STH epidemiology in India have been carried out in northern, western and southern parts of this country. Currently there is limited information available on the STH disease burden in the North-eastern states viz. Assam, Manipur, Mizoram, Arunachal Pradesh, Meghalaya, Tripura and Nagaland. This pilot study, was conducted among the children studying in government primary schools in those states in order to generate a preliminary estimate of current prevalence and intensity of STH which would help in formulating deworming frequency.

MATERIALS AND METHODS

2.1. Ethics, and Permission

Permission to the conduct the survey in selected schools had been obtained from Ministry of Health & Family Welfare, Govt. of India as well as of each individual states and also from Ministry of Secondary Education of each state. This cross-sectional study was designed according to WHO recommendations and approved by Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases (NICED), Kolkata. Written consent was obtained from the guardians prior to including the school children in the survey.

2.2. Study setting

The North-east of India originally comprises 7 sister states viz. Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Assam. As per 2011 census the total population of Northeast India is around 45 million.

2.3. Sampling procedure and sample size

For collection of data, WHO recommended Sentinel site Approach was followed. A list of randomly selected primary schools from both rural and urban areas in each state was prepared. Fifty students (age group 5-12years), who were present on the day of survey, were selected at random from each school.

2.4. Data collection

The study was conducted by Division of Parasitology, ICMR-NICED, Kolkata, during December, 2015- February, 2016. The field team demonstrated the aim, objective and procedure of fresh stool sample collection to the Head of the respective school and to the guardians of all the participants. Guardians and participants both were interviewed for relevant information like sanitation system, drinking water system, house condition, family income etc.

2.5. Sample collection

Fresh stool sample, defecated in the morning, was collected in a plastic screw cap bottle. Bottles were put in a zip lock bag and delivered to the temporary laboratory facility in ice box within an hour of collection.

2.6. Stool sample processing

The sample was processed using the Vestergaard Frandsen Kato-Katz kit following the manufacturer's protocol [5]. Two slides were prepared for each sample and were checked for the helminth egg. From the slide count egg per gram was calculated to measure the intensity of the worm infection.

2.7. Quality control

For the purpose of quality control, 10% slides were rechecked. An independent expert also reviewed the slides and survey procedure as well.

2.8. Data analysis

Excel 2010 were used to calculate the prevalence and to prepare graphical representation of the collected data.

RESULTS

The cross-sectional study, included 20 primary schools in 7 Northeast states, majority of which were in rural areas, with a source of drinking water and toilet facilities within the premises. A total of 1116 individuals was enrolled in this survey, out of which 1071 (96%) faecal samples were properly collected and transported for examination, following the sample acceptance criteria. Rest of the samples (45) were rejected as they did not pass the acceptance criteria.

All of the study participants were in the age group of 5-12 years. 531

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Original Research Paper

Physiotherapy

GENETIC VARIATION OF *GIARDIA LAMBLIA* LOCAL ISOLATES FROM KOLKATA, INDIA

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KEYWORDS :

INTRODUCTION

Giardia intestinalis, a protozoan parasite is causative agent of diarrheal disease in humans as well as large number of wild and domestic animals. This enteric parasite is considered a species complex having eight distinct assemblages (A-H).[1] They exhibit little differences in their morphology, but possess remarkable genetic variability. Among them members of only assemblage A and B cause human infection. [2] *G. lamblia* assemblage A is divided mainly into three sub-assemblages: AI, AII and AIII. Assemblage B is divided into two sub-assemblages, BIII and BIV. [3] The objective of this study was to identify inter- and intra-assemblage genetic variation in *G. lamblia* among patients in Kolkata and adjacent areas in eastern India. As of now there is very little information available on the diversity of Giardia sub-assemblages and multi-locus genotypes infecting people in this region. The genetic assemblages and subtypes were determined via multilocus sequence typing (MLST) using two house-keeping genes: -giardin and triose phosphate isomerase gene loci.

Materials and Methods

Study population and collection of faecal samples

A total of 413 faecal samples were collected from I.D. Hospital, Beliaghata, Kolkata between January, 2019 to May, 2019. Written consent from patients or guardians according to the availability were collected.

Microscopic examination of the faecal samples

All faecal samples were examined using both Lugol's iodine staining and wet smear technique under a compound microscope in of 400x magnification.

DNA isolation and molecular detection

DNA from the stool samples was extracted directly using Stool DNA Mini Kit (QIAGEN, USA) according to manufacturer's protocol. Molecular detection of *G. intestinalis* was achieved by amplifying 211bp of beta-giardin gene. The primer sequence used is as follows: GldtF: 5'-ATAACGACGCCATCGCGGCTCTCAGGAA-3', GldtR: 5'-TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA-3' [4]

The PCR mix consisted of 1X buffer containing 1.5 mM $MgCl_2$ (Roche, Germany), 200uM of each dNTP, 10 pmol of each primer (GCC Biotech), 2.5 units of Taq DNA polymerase enzyme (Roche) and 125ng of isolated DNA in final volume of 50ul. PCR cycle condition was: After an initial denaturation step of 5 min at 94°C, a set of 35 cycles was run, each consisting of 30 sec at 95°C, 1 min of annealing at 65°C and 60 sec at 72°C, followed by a final extension of 7min at 72°C and kept for hold at 4°C.

Amplification of beta- giardin and triose phosphate isomerase gene

The samples that were found positive for the presence of *G. intestinalis* in both microscopy and PCR were further used for multilocus genotyping.

A 511bp fragment of beta-giardin (bg) gene was amplified via nested PCR. The required primer pairs are: GlbgF1: 5'-AAGCCCGACGACCTCACCCGCAGTGC-3', GlbgR1: 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'; GlbgF2: 5'-GAACGAACGAGATCGAGGTCCG-3', GlbgR2: 5'-CTCGACGAGCTTCGTGTT-3' [4]

A portion of triose phosphate isomerase (tpi) gene with a fragment size of 530bp was amplified via nested PCR using 2 sets of primers viz. GltpiF1: 5'-AAATATGCCTGCTCGTCG-3', GltpiR1: 5'- CAAACCTTTCCGCAAACC-3'; GltpiF2: 5'-CCCTTCATCGGGGTAACTT-3', GtpiR2: 5'-GTGGCCACCACCCCGTGCC-3'.[4]

In both cases, The PCR mix consisted of 1X buffer containing 1.5 mM $MgCl_2$ (Roche, Germany), 200uM of each dNTP, 10 pmol of each primer (GCC Biotech), 2.5 units of Taq DNA polymerase enzyme (Roche) and 125ng of isolated DNA in final volume of 50ul.

PCR cycle condition for *bg* gene amplification was: After an initial denaturation step of 10 min at 94°C, a set of 30 cycles was run, each consisting of 30 sec at 94°C, 1 min of annealing at (65°C for primary, 55°C for nested) and 60 sec at 72°C, followed by a final extension of 5min at 72°C and kept for hold at 4°C.

PCR cycle condition for *tpi* gene amplification was: After an initial denaturation step of 10 min at 94°C, a set of 30 cycles was run, each consisting of 30 sec at 94°C, 1 min of annealing at (52°C for primary, 60°C for nested) and 60 sec at 72°C, followed by a final extension of 5min at 72°C and kept for hold at 4°C.

Agarose Gel Electrophoresis

After completion of PCR, the PCR products were electrophoresed in 1.5% agarose gel to observe specific bands in presence of marker (Figure- 1, 2).

Sequencing Reaction

The purified products of the nested PCR were then sequenced bidirectionally by using ABI PRISM®3100 Genetic Analyzer (Applied Biosystem) with following primers separately: GlbgF2, GlbgR2 for *bg*; GltpiF2, GltpiR2 for *tpi*.

Phylogenetic analysis

The sequences were validated using the BLAST database search and then aligned using ClustalW. Afterwards, a rooted phylogenetic tree was created via UPGMA method with clustalW. Representative sequences of *Giardia intestinalis* isolates from human were obtained from the NCBI database and were used to prepare the tree (Figure-3, 4).

Chapter 2

Visceral Leishmaniasis

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Additional information is available at the end of the chapter

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Abstract

Clinically, leishmaniasis is of three types—visceral leishmaniasis (VL) or kala-azar, cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). Post-kala-azar dermal leishmaniasis (PKDL) is considered as a complication of VL. VL is characterized by fever, anemia and splenomegaly in a VL-endemic area (malaria excluded). A subject with such symptoms should be subjected to an rK39 strip test. Confirmation of diagnosis is made by demonstration of the parasite (*Leishmania donovani*) from samples obtained by aspiration of bone marrow or iliac crest puncture. Miltefosine, stibogluconate, amphotericin B, liposomal amphotericin B and paromomycin are effective available anti-leishmaniasis drugs. Vector (*Phleblotomus argentipes*) control for reduction of transmission and early diagnosis and complete treatment are essential elements of case management. There is no effective vaccine against VL. This review on VL aims at providing state-art knowledge on epidemiology, diagnosis and case-management and vaccine development.

Keywords: leishmaniasis, PKDL, rK39 strip test, kala-azar vaccine

1. Introduction

The distinct clinical forms of leishmaniasis are visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). PKDL is considered to be a complication of VL. Kala-azar is a neglected tropical disease (NTD). It affects the poorest of the poor living in endemic areas. Post-kala-azar dermal leishmaniasis (PKDL) is associated with stigma. Fortunately, it is not difficult to diagnose the disease and several drugs are available for treatment of the disease.

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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].

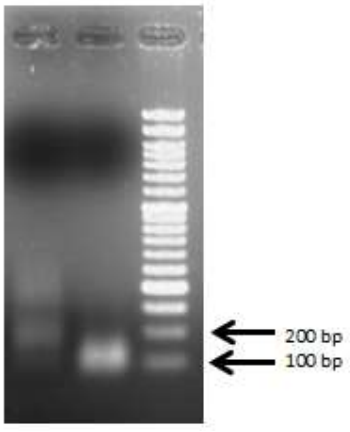

Figure 1: Nested PCR showing 110 bp *Naegleria* specific band (lane 2).

The disease is very serious and often fatal [2]. The patient unfortunately died 10 days after admission. The disease was first