CHAPTER-1
INTRODUCTION
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INTRODUCTION

Although there is a general feeling that misdiagnosis is quite often, with many people giving unreliable accounts of their own experiences, it is however difficult to get exact data. Although there are many studies of adverse drug events but there is a relative lack of misdiagnosis studies.

1.1 Historical relevant: a glimpse:

Amoebiasis as a deadly disease may have been first recognized by Hippocrates (460 to 377 B.C.), who first described a patient with fever and dysentery. Later (140 to 87 B.C.), the Old Testament and Huang Ti’s Classic in Internal Medicine made reference to dysentery (Kean, 1988). It was Feder Losch in 1875 who first identified Entamoeba histolytica in human faecal samples and was considered to be associated only with inflammatory process and accordingly named Amoeba coli (Lesh, 1975). Later in 1903 differentiation of Entamoeba histolytica and Entamoeba coli was established by Fritz Schaudinn and documented its taxonomic description with the name of E. histolytica based on its ability to cause tissue lysis (Schaudinn, 1903).

In 1925, E. histolytica was differentiated from its closely related species E. dispar, which was proposed to be non pathogenic and found only in asymptomatic carriers (Brumpt, 1925). However it was ignored for 50 years till the biochemical evidence in favour of sub groups within E. histolytica was reported in 1973, based on differences in lectin agglutination properties of parasites isolated from amebic patients and asymptomatic individuals (Martinez-Palomo et al., 1973). With the development of axenic culture medium for E. histolytica by Louis diamond in 1960 further allowed in vivo and in vitro studies (Diamond et al., 1961) and subsequently E. histolytica strains were distinguished by isoenzyme electrophoresis by Sargeaunt and Williams, thus confirming that E. histolytica was indeed a species complex comprising a pathogenic and non-pathogenic species (Sargeaunt et al., 1978). Brumpt's original 1925 hypothesis, was re-described, concluding that there was enough evidence to support the existence of species complex comprising a pathogenic E. histolytica and nonpathogenic E. dispar.
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(Diamond et al., 1993). Later in 1997, the World Health Organization accepted this hypothesis (WHO, 1997).

1.2 *Entamoeba histolytica*: The etiologic agent of amoebiasis:

Amoebiasis also called Traveller’s diarrhea is defined by the World Health Organization and Pan American Health Organization (PAHO) as infection with an enteric protozoan parasite *E. histolytica*, regardless of symptomatology. The causative protozoan parasite, *E. histolytica*, a fecally-orally spread pathogen in its trophozoite stage secret proteinases that dissolve host tissues, thereby kill host cells on contact, engulf red blood cells and invade the intestinal mucosa, causing amoebic colitis. In some cases amoebas burrows through the intestinal wall and travel through the portal vein to the liver causing amoebic liver abscess (ALA) consisting of *E. histolytica* trophozoites surrounding dead and dying hepatocytes and cellular debris contributing a significant cause of worldwide morbidity and mortality (WHO, 1997).

In accordance with the WHO report, about 40-50 million people are infected annually causing approximately 100,000 deaths place amoebiasis second only to malaria in causes of mortality worldwide (WHO, 1997). Most infections (≥ 90%) remain asymptomatic; suggesting that invasion of tissue is a peculiarity rather than a typical behavior of *E. histolytica* while the other 10% develop clinically overt disease (Fotedar et al., 2007a). The target organ primarily colonized by the parasite is the sigmoid and colon mucosa, where only invasive strains of the species *E. histolytica* can present their invasive potential producing tissue damage (Pritt and Clark, 2008).

Clinical manifestation of the infection is abdominal pain along with bloody diarrhea and colitis. The parasite can invade other organs, especially liver which may further result into liver abscess. The parasite thrives mainly in places with poor sanitation and such a condition being prevalent in developing countries, amoebiasis is a major problem of developing countries. The natural host of the parasite is human and other non-human primates. It infects people irrespective of sex and age; however, geographic location, socio-economic status, bad hygiene, host susceptibility and differences in strain specific virulence patterns greatly affect the risk of infection (Nichols, 2014). High reported prevalence in people in highly endemic areas probably due to recurrent asymptomatic infections while in developed countries, infection is
most commonly found in travelers from countries where the disease is an endemic one, institutionalized person, and patients infected with human immunodeficiency virus (Herbinger et al., 2011).

1.3 *Entamoeba histolytica*: An early branching amitochondriate anaerobic protozoan parasite:

It belongs to domain- Eukaryota; phylum- Amoebozoa, and order- Amoebida. In general parasites belonged to genus *Entamoeba* are unicellular eukaryotes that parasite vertebrates, invertebrates and possibly other unicellular eukaryotes. Amoebiasis is highly prevalent in developing countries because of impure water, low levels of sanitation, open air defecation, overcrowding, low socio-economic status, coupled with low literacy rates of parents. The parasite has two stages in life cycle; the invasive, active trophozoite and the inactive infective cyst. The nucleus is spherical in shape covered by a bilayered nuclear envelope with numerous pores while the evenly distributed chromatin clumps found inside the nuclear membrane are usually uniform in size (Clark, 2000). Unlike other eukaryote it typically lacks membrane bound organelles like golgi complex, mitochondria and rough endoplasmic reticulum. However, the presence of double membrane bound DNA containing mitochondria-like organelles, differently called “crypton” (Mai et al., 1999; Ghosh et al., 2000) and “mitosome” (Tovar et al., 1999) have been reported.

1.4 *Entamoeba histolytica*: the life cycle and the disease:

1.4.1 Life cycle: The parasite has a relatively simple oro-fecal life cycle without the involvement of any intermediate host (Figure 1.1). The parasite has a two stages life cycle, the non-motile infective stage known as cyst, and the invasive, and a multiplying stage known as trophozoite. *E. histolytica* cysts are round, tetra-nucleated, usually 10-15 µm in diameter and are surrounded by a wall made of chitin (Clark, 2000). The uninucleated motile trophozoites are pleuromorphic in shape, measuring about 10-50 µm in diameter. Infection occurs with the ingestion of food and water contaminated with fecal material of infected persons. Excystment occurs in the terminal ileum, where it give rise to eight daughter trophozoites and colonize the host’s tissues. Trophozoites move by extending creeping projections of cytoplasm, called pseudopodia, which pull them along and start encystment as they move further down the large intestine. Cysts
are then passed with the feces and may remain viable for weeks to months under damp conditions.

**Figure 1.1:** Life cycle of *Entamoeba histolytica* a) Mature cyst stained with 4% Lugol solution (100X magnification). b) Mature cyst without staining (100X). c) Trophozoite observed with differential interference contrast (DIC) (100X). d) Trophozoites of *E. histolytica* species with phagocyted erythrocytes (DIC 40X) (Ximenez et al., 2011).

Inside humans *E. histolytica* lives and multiplies as trophozoites. Amoebae typically live on a diet of intestinal bacteria and host food debris within the large bowel and can persist there for months and years causing only an asymptomatic luminal gut infection. The invasive form trophozoite can penetrate the intestinal mucosa causing “amoebic colitis” and dysentery, and occasionally disseminate to other organs, such as liver, lungs or brain where they where induce abscess formation. These are usually fatal if left untreated or wrongly treated. Tissue invasion is not part of the life cycle as those amoebae that pass across the mucosa are no longer capable of giving rise to new
infections. Invasive disease must therefore be viewed as aberrant behaviour on the part of the organism.

**1.4.2 Clinical presentation of the disease:**

Infection ranges from asymptomatic colonization of the large intestine to severe invasive intestinal colitis and extra-intestinal disease.

**1.4.2.1 Asymptomatic colonization:**

Most infections up to 90% remain asymptomatic or symptoms are very mild, suggesting that tissue invasion is a peculiarity rather than a typical behavior (Jackson et al., 1985; Gatti et al., 2002; Watanabe et al., 2014b). Asymptomatic carriers have normal rectosigmoidoscopic findings, without a history of blood in stool samples, absence of haematophagous amoebic trophozoite or Charcot-Leyden Crystal (Garcia and Bruckner, 1997). Interestingly, asymptomatic infection with *E. histolytica* but not *E. dispar* is associated with positive serum anti-amebic antibody responses and, frequently, a positive stool antigen test even in the absence of invasive disease (Abd-Alla et al., 1998).

Although infection with these amoebae is much more common than with *E. histolytica*, so far especially in developed countries *E. dispar* has never been known to cause colitis or amoebic liver abscess. Presently, diagnosis of intestinal amoebiasis in many countries relies commonly on stool microscopy for the presence or absence of cyst or trophozoite stage, it is difficult to figure out the actual percentage of asymptomatic patients infected with *E. histolytica* (Krogstad et al., 1978). However, if left untreated asymptomatic colonisation can lead to intestinal or extra intestinal colitis and it is estimated that 4-10% of asymptomatic individual present signs and symptoms associated with invasive amoebiasis (Haque et al., 2001). Estimation of the true prevalence of amoebiasis is not easy, because many studies were done with microscopic examination of a stool sample only which is often associated with false positive as well false negative result (WHO, 1997; Jackson, 2000). Moreover, asymptomatic *E. dispar* colonisations do not show evidence of serum anti-amoebic antibody response and disease compare to symptomatic *E. histolytica* intestinal infection which does show a systemic immune response (Gathiram and Jackson, 1987).
1.4.2.2 Intestinal colitis and dysentery:

Approximately, 10% of individuals infected with *E. histolytica* will develop clinical symptoms with intestinal or extra-intestinal involvement (Stanley, 2003). Clinical syndromes associated with intestinal *E. histolytica* disease include diarrhea, acute rectocolitis, toxic megacolon, fulminant colitis with perforation, ameboma, chronic nondysenteric colitis, and perianal ulceration. The onset of acute rectocolitis is usually gradual over 1 to 3 weeks. Most patients have abdominal pain, tenderness, watery or bloody diarrhea while only one third are febrile. Diarrhea can occur with up to 10 (or even more) bowel movements per day, and fever occurs in one-third of the patients (Reed, 2000). Patients often unwilling to eat and one fifth develops weight loss. Patients may have heme-positive stools (Charcot-Leyden crystals); presence of blood in stool, but fecal leukocytes may not be present in the acute stage.

Clinical diagnosis of amoebiasis is difficult because of the non specific nature of symptoms as it is easily confused with shigellosis and a number of other bacillary dysenteries (*Salmonella, Campylobacter*, enterohemorrhagic and enteroinvasive *Escherichia coli*) that are prevalent in tropical and sub-tropical countries (Haque et al., 1997; Sakata et al., 2001). In addition, it is very difficult to differentiate the symptoms of non-infectious intestinal diseases (ischemic colitis, inflammatory bowel disease, diverticulitis, and arteriovenous malformations) from infectious diseases because of the lack of fever in patients with amoebic colitis (Dunzendorfer and Kasznica, 1998). Moreover, chronic non-dysenteric intestinal amoebiasis characterized by intermittent diarrhea, flatulence, presence of seropositivity, and amoebae in the stool, can be puzzled with ulcerative colitis, resulting in misdiagnosis and subsequent treatment with corticosteroids (Ravdin, 2000). A generalised algorithm for the diagnostic approach to intestinal amoebiasis is represented in Figure 1.2.

Colonic findings in amoebiasis can vary from thickening of the mucosal wall to flask-shaped ulcer in colon (Garcia and Bruckner, 1997). Complications of intestinal amoebiasis include development of fulminant colitis (Ishioka et al., 2011; Arora et al., 2012; Kawazoe and Nagata, 2012), ameboma (Fernandes et al., 2009; Lin and Kao, 2013), cutaneous amoebiasis (Kenner and Rosen, 2006; Fernandez-Diez et al., 2012) and fistulas (Lysy et al., 1991; Jones et al., 2011).
1.4.2.3 Extra-intestinal amoebiasis:

Amebic liver abscess is the most common extra-intestinal manifestation of amoebiasis and is 10 times more common in adult men despite an approximately equal sex distribution of colonic amebic disease. Approximately 80% of patients manifest relatively quickly, typically within 2 to 4 weeks (Haque et al., 2003a). The acute symptoms include fever and a constant, dull, aching pain in the right upper quadrant or epigastrium while a subacute course may present with prominent weight loss with less fever or abdominal pain (Adams and MacLeod, 1977). Hepatomegaly with point tenderness over the liver below the ribs or in the intercostals spaces is a typical finding.
Right-sided pleural pain or referred shoulder pain occurs when the diaphragmatic surface of the liver is involved.

ALA associated gastrointestinal clinical symptoms include elevated peripheral white blood cell counts, alkaline phosphate levels (Nazir and Moazem, 1993; Saleem, 2009; Sarda et al., 2011; Otto et al., 2013). Complications associated with extra intestinal amoebiasis include direct extension from the liver to the pleura, pericardium, brain abscess and genitourinary amoebiasis. Pleuropulmonary amoebiasis is the most common complication of amebic liver abscess occurs as a result of the rupture of a superior right lobe abscess with erosion through the diaphragm to involve the pleural space (Blackett, 1988; Ozbay et al., 1997; Shrestha et al., 2010; Chandra et al., 2013). Genitourinary amoebiasis is rare and includes rectovaginal fistulas and vulvar lesions in women and penile amoebiasis in men (Mayhew et al., 2000). Cerebral amoebiasis has abrupt onset, and progresses rapidly to death over 12-72 hours without adequate therapy and generally observed in a small percentage of patients with ALA (De Villiers and Durra, 1998; Viriyavejakul and Riganti, 2009; Petri and Haque, 2013).

Lack of a history of intestinal disease within 1 year in many patients and lower sensitivity of serologic analysis together make amoebic liver abscess diagnosis very difficult (Adams and MacLeod, 1977; Maltz and Knauer, 1991; Johnson et al., 1994). In addition, ultrasonography, abdominal computed tomography, and magnetic resonance imaging are incapable of distinguishing amoebic from pyogenic abscesses (Adams and MacLeod, 1977). The definitive diagnosis of ALA involves positive serological tests for antibodies specific to *E. histolytica* and manifestation of hepatic lesion by imaging techniques like computed tomography ultrasonography, magnetic resonance imaging, and technectium-99 liver scan (Tanyuksel and Petri, 2003), fine needle aspiration (Mokhtari and Kumar, 2014).

1.5 Pathogenicity:

This monogenetic parasite is unique among the intestinal amoeba because of its ability to invade host tissue. Although recent studies provide new insights into the mechanism of pathogenesis, many questions yet to be answered particularly the trigger mechanism that converts the commensal dwelling organism into a tissue invasive pathogen. However, it is now well established that the host immune response plays a significant
role in the development and progression of disease but again little is known about the components of parasite that actually commence the cascade. It was found that a number of virulence factors involved in the course of pathogenesis such as N-acetyl-D-galactosamine-specific lectin (Petri et al., 1987; McCoy et al., 1993), amoebapore protein (Lynch et al., 1982; Rosenberg et al., 1989), cysteine protease (Keene et al., 1986; Keene et al., 1990) and cysteine peptidases (Matthiesen et al., 2013). Gal/GalNAc inhibitable lectin secreted on the surface of parasite is required for the adhesion of the parasite to host cells. The parasite binds to the Gal and GalNAc residues on mucus glycoprotein by using their surface adherence N-acetyl-D-galactosamine-specific lectin. This adhesion is almost completely inhibited by β-D-galactose (Gal), although some binding still occurs at high concentrations of Gal, indicating that other molecules viz., a 220 kDa membrane protein, a serine-rich protein and a cysteine protease–adhesin dimer are also participating (Laughlin et al., 2005). In addition the parasite also elaborates a number of enzymes, including membrane-associated neuraminidase and glucosaminidase, sialidase and calmodulin like calcium binding protein EhCaBP (Que and Reed, 1997; Udezulu and Leitch, 1987; Werries et al., 1983; Nok and Rivera, 2003; Sahoo et al., 2004; Aslam et al., 2012).

Binding of *E. histolytica* to the host cell is rapidly followed by cell death and this is majorly caused by two processes permeablisation by pore forming peptides (Leippe, 1997; Leippe et al., 2005) and the induction of apoptosis (Seydel et al., 1998; Huston et al., 2000; Boettner et al., 2005). The other factor which plays important role in pathogenesis is cystein protease (Ankri et al., 1999; Lauwaet et al., 2003) due to its capability to dissolve intracellular matrix and hence tissue invasion. Recently, it has been found that cysteine proteases along with human tissue factors are required for degradation of extracellular matrix (Thibeaux et al., 2012).

It circumvents the host immune response through cleavage of secretory immunoglobulin A (sIgA), IgG, activation of complement (Campell et al., 1997; Que et al., 1997; Ravdin, 1988; Reed et al., 1993), IL-1B, IL-8, cyclooxygenase (COX)-2 (Stenson et al., 2001; Gutierrez-Alarcon et al., 2006). The final step during the invasion process is secretion of cytokines with multiple effects, including attracting neutrophils and macrophages by host epithelial cells in response to parasite invasion (Eckmann et al., 1995). Comparison of transcription profiles of virulence associated factors of
virulent and avirulent *E. histolytica* revealed that a number of genes were downregulated in avirulent species. Gene serine, threonine, and isoleucine rich protein (EhSTIRP) was shown to be essential for virulence as its downregulation in the virulent *E. histolytica* caused decrease in virulence (MacFarlane et al., 2007).

Comparison of some essential virulence factors involved in several amoebic functions among the amoebic strains having different degree of virulence revealed that amoebic virulence cannot be described completely by only a defined set of proteins. Rather it is a combinative process of multiple factors that are required for complete pathogenesis. If any of these functions are lacking, the pathogenesis will be diminished as in the case of *E. dispar* and *E. histolytica* Rahman strain (Olivos-Garcia et al., 2009). The existence of inherently avirulent strains has been suggested on the basis of a unique pattern of tRNA linked short tandem repeats (STR) (Escueta-de Cadiz et al., 2010). The Rahman strain of *E. histolytica* is considered to be avirulent in axenic culture as it shows reduced cytopathic activity on epithelial cells and also does not form liver abscesses in animal models (Ankri et al., 1999; Dvorak et al., 2003). However, an *E. histolytica* genotype specific virulence property is yet to be established.

Trogocytosis had previously been seen only in immune cells (Joly and Hudrisier, 2003) but the phenomenon has now been found in *E. histolytica*, where they kill host cells by ingesting distinct pieces of living human cells rather than ingesting the whole cell (Guillen, 2014). Cell death occurs because of elevation of intracellular calcium level. Thus ingestion of fragments of human cell is the key factor behind cell killing and intestinal tissue invasion. However, the phenomenon differs from what happened in case of immune cells as the phenomenon of trogocytosis observed in case of amoeba results in death (Ralston et al., 2014).

1.6. Genome organization of pathogen *E. histolytica*:

Karyotyping of *E. histolytica* using pulse field gel electrophoresis demonstrated that the number of chromosomes was 31-35 with size ranging from 0.3 Mb to 2.2 Mb (Willhoeft and Tannich, 1999). Further molecular analysis using cDNA probes established that there are 14 linkage groups which hybridized to as many as four chromosomal bands indicating functional ploidy of 4 in *E. histolytica* (Willhoeft and Tannich, 1999).
The 21Mb genome of *E. histolytica* was reassembled and reannotated in 2010 (Lorenzi et al., 2010) from the previous version (Loftus et al., 2005; Clark et al., 2007). The genome is very A+T rich (75%) and codes for 8333 annotated genes and displays a relatively low level of nucleotide diversity across its genome (Lorenzi et al., 2008; Weedall et al., 2012). The most updated information of genome sequence is available on https://www.amoebadb.org (Aurrecoechea et al., 2011). The other relevant information from genome sequence data is summarized in Table 1.1.

Besides chromosomal DNA, interestingly *E. histolytica* carries circular DNAs of various sizes varying from 5 kb to 50 kb (Lioutas et al., 1995; Dhar et al., 1995). The most copious are the 15-26 kb ribosomal episomes with a copy number of around 200 per haploid genome (Bhattacharya et al., 1998). The functions of other circular DNAs are not known, and these have not been sequenced. The best-characterized circular rDNA molecule in *E. histolytica* is the rDNA plasmid called EhR1 found in strain HM-1: IMSS. This circular rDNA plasmid encoding rRNA is 24.5 kb in size present in about 200 copies per haploid genome and has been fully sequenced (Sehgal et al., 1994; Bhattacharya et al., 1998).

Each molecule of EhR1 (Figure 1.3) contains two inverted rRNA transcription units (rDNA I and rDNA II). The two units were interrupted by a 3.7 Kb downstream intergenic spacer (HMd) and a 9.2 Kb upstream spacer (HMe and HMg). The downstream spacer is composed of two families of short tandem repeats; the 170bp DraI repeat and the 144 bp ScaI repeats whereas, the upstream spacer region contains several repeat families and are six ScaI repeats, eleven 145 bp PvuI repeats, two Hinfl repeats which share regions of sequence identity with DraI repeats of the downstream spacer, AvaII repeats and 74 bp repeats. The upstream spacer region also contains several stretches of unique sequence like Tr which is transcribed into a polyadenylated 0.7 kb RNA. However, this RNA lacks any significant open reading frame and its function is unknown (Bhattacharya et al., 1998). The overall organization of EhR1 in terms of rDNA unit size and families of repeats in intergenic spacers shows similarity to rDNA of other eukaryotes. Recent studies highlighted the presence of a variant of EhR1 molecule called EhR2 in strain HM-1: IMSS. Probably, this 14 kb molecule forms due to intramolecular recombination of direct repeats in EhR1, resulting in two
half molecules and only the molecule containing the HMe region is retained by the cell and is called EhR2 (Figure 1.4) (Ghosh et al., 2001).

**Table 1.1** Genomic features and annotation comparison of the pathogen *E. histolytica* (Lorenzi et al., 2010).

<table>
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<th>Old E. histolytica assembly</th>
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Figure 1.3: Ribosomal DNA circles from *E. histolytica* strains HM-1: IMSS (EhR1) (Bhattacharya et al., 1998).

Figure 1.4: Sequence organisation of the *E.histolytica* HM-1: IMSS rDNA plasmid EhR2. (Ghosh et al., 2003).

1.7 The *Entamoeba* complex and need for molecular surveillance:

While years of scientific discussions have left Brump's theory behind prior to the 1990s no molecular technology allowed clear differentiation of pathogenic *E. histolytica* and non-pathogenic *E. dispar* (Ximenez et al., 2011). The knowledge of these two species and a third species of *Entamoeba*, *E. moshkovskii*, with different pathogenic phenotypes comes from large scientific debates during the second half of the 20th century, which gave place to rapid development of molecular and immunological based diagnostics.
technology and subsequently has been detected in individuals living in endemic areas of amoebiasis (Ali et al., 2003; Parija and Khairnar, 2005; Khairnar et al., 2007; Fotedar et al., 2008; Anuar et al., 2012a) and could be contributing to the prevalence figures. The re-classification of *E. histolytica* into *Entamoeba* complex has further added to the complexity of the epidemiology of amoebiasis since they cannot be differentiated by microscopy, the most routinely used clinical diagnostic method, particularly in developing countries where the disease is an endemic one.

Traditionally, features like size of the trophozoites and cysts and the number of nuclei in the mature cyst are the basis for description of *Entamoeba* species in clinical settings (Figure 1.5). Thus, so far as epidemiology of amoebiasis is concerned, it remains uncertain, as most of the reported data were obtained using microscopy which is incapable in distinguishing *E. histolytica* from its two morphologically identical non pathogenic species.

<table>
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<tr>
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<th>Cyst</th>
<th>Tropozoite</th>
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<tr>
<td><em>E. histolytica</em>/</td>
<td></td>
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<tr>
<td><em>E. dispar</em>/</td>
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<td><em>E. moskovskii</em></td>
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<td><em>E. hartmanni</em></td>
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**Figure 1.5:** Cysts and trophozoites of *Entamoeba* species (Fotedar et al., 2007a).
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1.8 Stool and blood sample based diagnosis of amoebiasis:

The main purpose of detection and differentiation of *E. histolytica* species in stool samples is the detection of the causative agent of amoebic dysentery. However, major problems with a wide range of imperfect tests for the diagnosis of amoebiasis severely limit the understanding of its magnitude and epidemiology. A greater difficulty is the varied, inconsistent application of existing methods in different areas of the world. Since the differentiation of pathogenic *E. histolytica* from its nonpathogenic morphologically indistinguishable *Entamoeba* species, a number of methods viz., isoenzyme analysis particularly hexokinase after cultivating the parasite from cyst-positive fecal material, antibody based detection assay (indirect hemagglutination, latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis, the amebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay, and enzyme-linked immunosorbent assay), antigen heavy subunit of the galactose/N-acetyl-galactosamine inhibitable lectin based detection assay (TechLab ELISA kit), DNA blotting, PCR-based assays, including gene amplification with specific primers, multiplex PCR, restriction fragment length polymorphism, real-time PCR and microarray has been adapted for accurate detection of *E. histolytica* which is almost impossible using the conventional microscopy based diagnosis.

In the post-genomic era new diagnostic tools specific to *E. histolytica* are being exploited by clinicians and researchers to differentially identify and treat patients as well as to add to the knowledge of the epidemiology, natural history and transmission of infection. Studies suggest that PCR assay should be useful as a reference test for sensitive and specific differentiation of species of *Entamoeba* complex (Gonin and Trudel, 2003). Study conducted in Ethiopia and Nicaragua, PCR results showed that *E. histolytica* is a rare finding in patients with diarrhea (Kebede et al., 2004; Leiva et al., 2006). Therefore, it was concluded that at the health centers, *E. histolytica* was clearly over-diagnosed when microscopic methods were employed.

1.9 Impact of HIV and environmental factor on prevalence of amoebiasis:

It is now well accepted that HIV infection has been modifying both the epidemiology and outcome of intestinal parasitic infections. Diarrhea is an important clinical problem among HIV-infected patients and hence associated with both morbidity and mortality.
Following the HIV/AIDS pandemic, numerous studies established that intestinal protozoan parasites such as Cryptosporidium sp, Microsporidia sp, Isospora belli, and Cyclospora cayetenensis were frequently associated with severe diarrhea in both industrialized and developing countries. However, there have been controversies regarding the impact of HIV on the occurrence of amoebiasis though recent sporadic reports made from Japan, Mexico, Taiwan, and South Africa shown significant association of E. histolytica infection and HIV patients (Moran et al., 2005a; Hung et al., 2008a; Samie et al., 2009; Watanabe et al., 2011; Watanabe et al., 2014a).

The burden of diseases associated with intestinal parasitic infections carried out in different countries have shown that environmental, socio-economic level, demographic and personal hygiene and sanitation greatly influence the transmission and distribution of intestinal parasitic infections (Norhayati et al., 2003). E. histolytica is an important protozoan intestinal parasite in resource-poor settings particularly in developing country like India where the disease is an endemic one. However, to the best of our knowledge no study were conducted in India to assess the importance of socio-demographic and other environmental factors on disease endemicity in this geographical region of the globe for further enriching our knowledge on transmission of E. histolytica infection.

1.10 Research gap in relevance to amoebiasis epidemiology in North East India:

The true epidemiology of E. histolytica, E. dispar, and E. moshkovskii infection still remains unclear due to their morphological identity. Most morbidity and mortality due to amoebiasis occur in developing countries such as Central America, Africa and the Indian subcontinent. Sporadic studies have been performed in India, but after the re-description of E. histolytica and E. dispar very few initiatives were taken to evaluate the true prevalence in Indian population (Parija and Khairnar, 2005; Srivastava et al., 2005). While, the report of colonization of E. moshkovskii in Indian population further suggested the importance of characterizing the Entamoeba complex in Indian population with the aim of better management of amoebiasis by avoiding unnecessary chemotherapy when the infection is in terms of nonpathogenic species.

Recent studies in the areas of endemicity have suggested that prevalence of infection by the invasive parasite E. histolytica and the non-invasive parasite E. dispar
and *E. moshkovskii* is not yet investigated from North Eastern region besides considering an area of endemicity. Prevalence study using DNA based diagnostic, a substantial amount of work has been done over the last decade across the world including sporadic areas in Indian context, but there is no initiation from North East India till date. Hence, the present molecular epidemiological study has been undertaken to evaluate the true prevalence of *E. histolytica* among the population of North Eastern region with special reference to flood plain area like Barak Valley from Assam and its neighboring areas using rDNA based PCR amplification of *E. histolytica* in stool samples considering its high sensitivity and specificity compared to other diagnostic techniques available so far.

1.11 Intestinal protozoan parasitic co-infection– a neglected reality:

Intestinal parasitic infections (IPIs) have a worldwide distribution and have been identified as one of the most significant causes of gastrointestinal morbidity, malnutrition and mortality worldwide. Epidemiological information on the prevalence of intestinal parasitic infections in different regions is a prerequisite to develop appropriate control strategies. According to WHO globally around 3.5 billion people are affected, and 450 million are sick because of these intestinal parasitic infections (WHO, 1998). It is estimated that as much as 60% of the world’s population are infected with intestinal parasites and are among the most common infections worldwide (WHO, 2002).

With regards to intestinal protozoan infections *Giardia duodenalis, Entamoeba histolytica, Cryptosporidium parvum, Blastocystis hominis, Cyclospora cayetanensis, Enterocytozoon bieneusi, Isospora belli* are the most predominant protozoan infection throughout the globe and they are associated with diarrhea (Haque, 2007). Although intestinal polyparasitism seems to be a common feature in human populations, but little is known about its epidemiological significance, long term impact on human health (Nguhiu et al., 2009). Indeed, it is routinely ignored in epidemiological surveys; whilst it is common that prevalence data are presented at a per-species basis, multiparasitism rates are seldom reported (Cox, 2001). There is a pressing need to extend our understanding of the epidemiology and magnitude of co-infection in particularly resource poor settings across the world, in order to accurately evaluate its true scale,
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geographical distribution and public impact. Such understanding is needed to aid an evidence based appraisal of the global load of multiparasitism to make justified claims for integrated control programmes.

1.12 Outline of the research problem:

The morphologically indistinguishable nonpathogenic *E. dispar* is common in humans in many parts of the world. Similarly *E. moshkovskii*, which was previously considered as a morphologically identical free-living amoeba now a days is highly prevalent in human population of *E. histolytica* endemic countries. In order to avoid unnecessary anti-amoebic chemotherapic treatment of individuals infected with non-pathogenic Entamoeba species viz., *E. dispar* and *E. moshkovskii*, it is important to detect these two Entamoeba species differentially from the pathogenic *E. histolytica*. In addition, the need of simpler and better identification of these infections in clinical setting is not only in the aspect of correct diagnosis for rapid treatment decision and care management in terms of designing of control strategy, particularly in resource poor endemic areas where misdiagnosis is quite common and unnecessary anti-amoebic chemotherapy, but also for a better understanding of the disease epidemiology in the human population.

In addition there is relatively lack of sensitive and specific screening tool for Entamoeba in stool samples for performing large scale epidemiological study particularly in resource poor setting. Besides an endemic region, so far the prevalence of amoebiasis in North East India is concerned; no attempt has been initiated to determine the true prevalence of *E. histolytica* and potential risk factors associated with it. Moreover, as compared to pervasiveness of intestinal parasitic co-infection particularly among amoebic dysentery cases, relatively few studies have actually focused on it and very little is known about its epidemiological implication.

1.13 Novelty of the research:

Establishment of economic, faster and replicable way of screening Entamoeba positive samples from a large number of stool samples will be helpful for its implication as a screening tool in epidemiological study particularly in resource poor setting where the disease in an endemic one. Little is known about the epidemiology of *E. dispar* and *E. histolytica*.
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*moshkovskii* infections in India, as only a few studies used molecular methods to identify this parasite. Given the important implications of both diagnosis and epidemiological studies for species differentiation of the *Entamoeba* complex and the fact that the prevalence of species specific infection is unknown, this study will assess the true prevalence of *E. histolytica*, *E. dispar* and *E. moshkovskii* infections in this remote corner of India and potential risk factors associated with the disease. Thus considering amoebiasis as a global health problem; the present study will obviously help in understanding the disease occurrence, its association with clinical, environmental and socio-demographic factors. Indeed, identifying predictors of *E. histolytica* infection is crucial for the effective implementation of control strategies in this endemic region of India. Furthermore, the present study will also help to understand the degree of co-infection of major diarrheal protozoan parasite with *E. histolytica* which will help in the development, validation and application of broad-spectrum diagnostic tools as well as designing integrated control strategy.

1.14 Objectives of the research:

In the era of post genomic, use of faster, affordable and reproducible diagnostic tools lead to a better understanding of the disease epidemiology. Moreover, being an endemic area, there are no data on the true prevalence and associated risk factor of *E. histolytica* infection from the North Eastern part of India. In addition, though intestinal parasitic co-infection seems to be a common feature in human populations in the region disease endemicity, very little is known about its actual magnitude. With this information, the objectives of the present study were-

1) Epidemiological survey and screening of stool samples for *Entamoeba* in the North Eastern states of India (Assam, Manipur, Meghalaya and Tripura).

2) *Entamoeba* spp. confirmation through Polymerase Chain Reaction and DNA sequencing.

3) To study the co-infection of other gastrointestinal Protozoan parasites following the oro-fecal route.

4) Study of incidence and prevalence of the parasite *Entamoeba histolytica* among the inhabitants under study.