CHAPTER 5

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5.1 Screening of stool samples for amoebiasis:

Infection with *E. histolytica* results in 34 million to 50 million symptomatic annual cases of amoebiasis worldwide, causing an annual deaths of 40 thousand to 100 thousand (WHO, 1997) with the highest incidence in South America, Africa and South East Asia, India being one of the endemic countries. The distribution pattern and increasingly frequent report of *E. histolytica* as water and food borne parasite suggests development of rapid and accurate identification methods for public health efforts to manage the disease. Various techniques are being followed for specific identification of *E. histolytica* in stool specimens such as microscopy, culture, zymodeme analysis, ELISA, PCR etc.

The pathogenic amoeba *E. histolytica* is indistinguishable in its morphic stages cyst and trophozoite from those of non-pathogenic *E. dispar* (WHO, 1997) and *E. moshkovskii* (Ali et al., 2003), except in rare cases of persistent disease when *E. histolytica* trophozoites may contain ingested red blood cells. The limitations of detection methods are their low sensitivity and inability to differentiate closely related species. The sensitivity of light microscopy is limited to only 60% at its best but confounded with false-positive results due to misidentification of macrophages and nonpathogenic species of *Entamoeba* (Haque et al., 1995). In addition, factors such as delays in the stool specimen processing and initiation of anti-amoebic treatment prior to sample collection can lead to *E. histolytica*-negative culture results even in those patient stool samples showing cysts/trophozoites by light microscopy (Parija and Khairnar, 2007).

A recent study highlighted the failure of TechLab ELISA kit, the most commonly used screening tool in detection of *E. histolytica* in any of the *E. histolytica* PCR positive samples in the United Arab Emirates (Elbakri et al., 2013). Most of studies till today were based on microscopy or ELISA as screening tool hereby performing the PCR confirmation on either microscopy or ELISA positive samples though both the techniques have conflicting result in terms of sensitivity.
The circular extra chromosomal rDNA episomes are highly abundant approximately 200 copies per cell (Bhattacharya et al., 1989; Huber et al., 1989; Sehgal et al., 1994) in \textit{E. histolytica}. This makes rDNA a significant molecular marker for sensitive detection of \textit{Entamoeba} DNA directly from stool samples and liver abscess samples. Different regions of rDNA molecule have been used for \textit{Entamoeba} detection (Samuelson et al., 1989; Bracha et al., 1990). Since major portions of the rRNA genes are conserved across species (Paul et al., 2002), the complete rRNA genes were not useful as diagnostic probes or primers.

Through a systematic approach for detection of \textit{Entamoeba}, we have been able to establish a cost effective and high throughput sensitive technique for screening large number of stool samples at a time. The dot blot analysis could screen \textit{Entamoeba} positive stool samples, maximum 30 samples at a time in a 96 well plate when samples are loaded in triplicate. Blind study conducted over 130 stool samples to test the sensitivity and specificity of microscopy, culture and the HMe probe to screen \textit{Entamoeba} positive samples in our population dot blot technique showed a sensitivity of 94% [77.19-98.95] and specificity of 100% [96.38-100.00] which is much higher than microscopy (62.07%, 77.23%) and culture (34.48%, 88.12%), implying its importance as a screening tool in epidemiological studies particularly in resource poor setting where the disease is an endemic one. Because of its good sensitivity we have been able to find out the true prevalence of \textit{E. histolytica} among the study population which will not be possible otherwise if we use only culture or microscopy as a screening tool (Figure 5.1). This technique saves the cost of two important steps which is very relevant in developing diagnostics for parasites in endemic countries like India.

The screening technique:

1. Minimizes the cost of large number of stool kits for isolating genomic DNA from the stool samples
2. Helps to scale down the number of subsequent PCR reactions to identify the parasite at the species level.
3. Solves the poor sensitivity associated with screening technique like microscopy, culture and ELISA making PCR analysis of samples those were microscopy, culture or ELISA negative.
In our study, of the 242 samples that were microscopically positive, 56 were *E. histolytica* and 84 were mixed infections with *E. histolytica*. Thus, only 57.8% of the samples, resembling *E. histolytica* by microscopy, were true *E. histolytica*, implying that remaining 42.2% of so-called infections were in terms of other two *Entamoeba* spp. Kebede et al. (2004) highlighted 91.4% of the microscopy positive samples were *E. dispar* among prisoners and primary-school children in Ethiopia. In another study conducted among 746 gastroenteritis patients attending Jawaharlal Institute of Postgraduate Medical Education and Research Hospital, Pondicherry, India, found only 19% of the 68 stool samples, resembling *E. histolytica* by microscopy, were actually *E. histolytica* (Parija and Khairnar, 2005). Similarly, in a report from Australia, fifty percent of the microscopy positive stool samples were positive for nonpathogenic *E. moshkovskii* in PCR assay (Fotedar et al., 2008). The negative PCR result in 18 microscopy positive stool samples is probably because of the presence of other *Entamoeba* species. However, this needs further studies using molecular tools to validate the existence of other commonly found *Entamoeba* species infecting humans.

**Figure 5.1:** Sample showing positive result in different screening tool employed in this study (Eh= *E. histolytica*).
5.2 Amoebiasis and co-infection of major protozoan parasite among amoebic dysentery cases:

The primary objective of epidemiological studies on the prevalence of intestinal parasitic infection in different localities is to identify high-risk communities and formulate an integrated control strategy (Gelaw et al., 2013).

5.2.1 Prevalence and incidence of amoebiasis:

To be able to distinguish and detect *E. histolytica*, *E. dispar* and *E. moshkovskii* in stool samples is exceedingly essential for accurate diagnosis of amoebiasis and to figure out the true magnitude of pathogenic *E. histolytica* in a community. In the era of post genomics various DNA based molecular methods have been developed for accurate diagnosis.

Studies from different part of the world indicate that the prevalence of *Entamoeba* species in the stool varies greatly. In our study stool samples were tested from asymptomatic as well as symptomatic population. As shown by the results of the present study, *E. histolytica*/*E. dispar*/*E. moshkovskii* is prevalent in North Eastern states of India with an overall prevalence of 23.2%. Prevalence rate of the *E. histolytica* observed in our cross-sectional study conducted at the level of community and hospital using molecular technique was 13.7%. The prevalence rate of *E. histolytica* found in our study area was higher in comparison to the rate 9.15% reported from a study conducted on microscopy positive samples in rural communities in Malaysia using molecular approach (Ngui et al., 2012). Emile et al. (2013) reported a much higher prevalence rate of 54.5% among Kigali Institute of Education students in Kigali, Rwanda based on microscopy.

A study in Australia reported that 70.8% of patients were infected with *E. dispar*, compared to 4.5% of *E. histolytica* and 61.8% of *E. moshkovskii* (Foteder et al., 2007b). Lau et al. (2013) reported a prevalence rate of 13.2% and 9.9% for *E. histolytica* and *E. dispar* respectively in Orang Asli settlements in Malaysia using real time PCR conducted on microscopy positive samples. From Northern India *E. histolytica* prevalence rate of 8.4% was reported using molecular approach (Srivastava et al., 2005). A much higher *E. histolytica* and *E. dispar* prevalence rate of 69.6% and
22.8% respectively was reported using PCR assay among among children in Gaza, Palestine (Al-Hindi et al., 2005).

In compare to our finding, an incident rate of 3.16 per 100 PY in MSM and 0.68 per 100 PY in other risk group in Taiwan were reported by Hung et al. (2008a). Lowther et al., (2000) reported incident rate of 13.5 cases per 10,000 person-years among HIV-infected patients in the United States. ElBakri et al. (2013) reported an average *E. histolytica* incidence rate of 13.3% in the United Arab Emirates (UAE) using nested PCR as diagnostic tool.

### 5.2.2 Co-infection rate of major protozoan parasite among amoebic dysentery cases:

Because of their similar clinical presentations and since microscopic diagnosis of these parasites is neither sensitive nor specific a multiplex real-time PCR assay for detection of *E. histolytica, G. duodenalis* and *C. parvum* has been developed by Verweij et al. (2004) where, PCR assay confirmed more positive samples compared to microscopy revealing the significance of molecular characterization of these parasite over microscopic methods. Singleplex PCR used in our investigation detected 17 more infection compared to microscopy, minimizing the chance of false negativity.

In our study, we have found higher co-infection rate of *G. duodenalis* in amoebiasis cases compared to other diarrhoeagenic protozoan parasite (Figure 5.2). Noor Azian et al. (2007) reported 3.8% co-infection rate of *G. duodenalis* with *E. histolytica* in an aborigine community in Pahang, Malaysia. Co-infection of *E. histolytica* and *G. duodenalis* was reported from different parts of the worlds (Herbinger et al., 2011; Al-Harazi et al., 2013). Recently, Anuar et al. (2013b) found *T. trichiura* (soil-transmitted helminths) and *G. intestinalis* in 42.0% (7/17) of symptomatic *E. histolytica* positive subjects.

In a different study in Ethiopian population, multiple infections were found in 4.6% of the total examined subjects (Gelaw et al., 2013). Recently, a hospital based study in Shanghai, China, reported co-infection of *Cryptosporidium spp.* and *Enterocytozoon; Cryptosporidium* and *Giardia* where all the *Cryptosporidium*-positive specimens were *C. andersoni* and most *G. intestinalis* were assemblage C (13.49%)
(Liu et al., 2014). Study of Blackwell et al. (2013) highlighted an antagonistic relationship between Helminths and *Giardia* infection suggesting the importance of co-infection for infection risk and recovery dynamics.

**Figure 5.2:** Representative figure showing prevalence of amoebiasis, co-infection and associated risk factors among NE population of India.

### 5.3 Association of HIV status, socio-demographic profile and risk factor with amoebiasis:

Epidemiological studies carried out in different countries have shown that the socio-economic level of the society significantly affect incidence of intestinal parasitic infections; however there is lack of adequate information on the impact of HIV, socio-demographic and environmental factors on occurrence of amoebiasis specifically.
5.3.1 Clinical status of HIV patients and parasite associations:

We observed a significant fluctuation in the prevalence of the parasite on the basis of the HIV status of the patients. As expected, with the decrease in the CD4 cell count in the HIV positive patients, a significant increase in parasite load was observed, supporting the earlier observations made in Ethiopian, Mexican populations and Lao People’s Democratic Republic (Moran et al., 2005b; Adamu et al., 2013; Paboriboune et al., 2014). Our study further confirmed that antiretroviral treatment contributed significantly in protecting individuals from *E. histolytica* infection. Apart from CD4 cell count and ART, HIV sero-positive men who have sex with men in Taiwan are in increased risk for *E. histolytica* infection and invasive amoebiasis (Hung et al., 2008a). We did not find a significant correlation among HIV positive group with respect to *E. histolytica* infection and age group, perhaps due to non availability of sufficient samples in the different age group. Similarly, no significant correlation was observed with respect to amoebiasis and symptom of the subject perhaps antiretroviral therapy and anti-amoebic chemotherapy interferes to establish any significant correlation as most of the subjects become asymptomatic cysts carriers or passers.

While, considering the association of *Entamoeba* complex with CD4 cell count in the HIV positive patients we did not find any significant association with lower infection in subjects with CD4 counts 200-300 cells/µl which may be because of the start of antiretroviral therapy in this group further supporting the earlier report on the significance of antiretroviral therapy in the prevention of opportunistic parasitic infection (Bachur et al., 2008; Adamu and Petros, 2009; Missaye et al., 2013). Like *E. histolytica* infection we did not observe any significant association of *Entamoeba* complex prevalence and age group, the reason being non availability of sufficient samples in the different age group.

5.3.2 Association of socio-demographic profile and selected environmental factors with amoebiasis and *Entamoeba* complex:

Regression analysis revealed that the prevalence of *E. histolytica* was highest in the monsoon season followed by the pre- and post-monsoon seasons. This further indicated the high rate of faecal–oral contamination during the monsoon season, probably due to lack of safe drinking water. The prevalence was significantly high in the <15 or 16-30
years age groups. Studies from different geographical areas of the globe also reported that the intensity of intestinal parasitic infections including *E. histolytica* were significantly higher among children (Waqar et al., 2003; Sayyari et al., 2005; Zahida et al., 2010; Ortega and Sanchez, 2010; Ngui et al., 2011; Wegayehu et al., 2013). Results of the present study indicate a non-significant difference in the prevalence of *E. histolytica* infection between genders. Rivera et al. (1998) reported that there was a non-significant difference in the gender distribution of *E. histolytica* infection in 14 communities in the northern part of the Philippines. Similar observations have been reported from different parts of the world (Magambo et al., 1998; Hamze et al., 2004; Sharma et al., 2004; Zahida et al., 2010; Tasawar et al., 2010). However, most hospital-based studies reported gender predominance of *E. histolytica* infection. Ozyurt et al. (2007) reported 67% prevalence of *E. histolytica* in males and 33% in females among patients attending a training hospital in Turkey. Ohnishi and Murata (1997) studied the prevalence of *E. histolytica* in the area of Tokyo, Japan. Out of 28 study cases, 26 were males and none of the females were infected. On the other hand, prevalence rates of 64% (16/25) for *E. histolytica* infection in females and 36% (9/25) in males were reported among attendees of a health care centre in Turkey (Ozgumus and Efe, 2007). A hospital-based study in Pakistan observed a significantly high prevalence rate of *E. histolytica* infection in females (31.5%) compared with males (19.6%) (Ejaz et al., 2011).

The association between infection and occupational status indicated that student/ pre-school and daily laborers including farmer, driver were the two groups presented a more than two fold risk increase compared to Gov’t employer perhaps these are the two groups those intake street food. In our study, there was significant link between *E. histolytica* infection and participants’ level of education. This findings did not corroborate the results reported by Siddiqui et al. (2002) and Pham Duc et al. (2011). This present study also observed encouraging trends that individuals from the Muslim religion have a significantly greater risk of being infected by *E. histolytica* compared with subjects from the Hindu and Christian religion. This was attributed to the poor housing condition, poor provision of basic amenities and poor sanitary practices. In addition rural background of respondents was also significantly associated with *E. histolytica* infection. As shown by other previous studies, our study further
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confirmed a tendency of high risk *E. histolytica* infection among the rural population, where prevailing poverty, poor socioeconomic status, low standards of sanitation and hygiene and use of local ponds or wells for day to day activities may contribute to high Entamoeba prevalence (Norhayati et al., 2003; Ngui et al., 2011).

Since Entamoeba may exist both as symptomatic as well as in asymptomatic state in the intestine, therefore, we concentrated on asymptomatic individuals. We found significantly higher prevalence of *E. histolytica* infection in symptomatic subjects compare to asymptomatic subjects. This finding is parallel with a study conducted in Malaysia (Anuar et al., 2013b), Turkey (Ozer et al., 2011), Netherlands (Visser et al., 2006) and Sweden (Lebbad and Svard, 2005). Similar correlation between clinical symptoms and *E. histolytica* infection was also observed in a study conducted in Mexico (Redondo et al., 2004). However, according to a recent review asymptomatic cyst passage, with 90% of human infections either asymptomatic or mildly symptomatic, is considered to be the most common manifestation of *E. histolytica* though the conclusion was based on studies that have been made by stool microscopy (Fotedar et al., 2007a).

The trend emerging from our analysis indicates that the various risk factors like toilet facilities, living conditions, hygienic practices, sources of drinking water, family history of infection, not washing hands always before taking food and previous history of anti-amoebic treatment are important predictors of *E. histolytica* infection. Such a correlation was also drawn from a study conducted in an agricultural community of Vietnam and in rural residents of Kenya (Pham Duc et al., 2011; Kinuthia et al., 2012). Abossie and Seid (2014) also highlighted significant association of educational status of the household heads, absence of washing facility, home cleanliness condition intestinal parasitic infections (IPIs) among primary school children in Chencha town, Southern Ethiopia. Significant association of diarrhea and not washing hands has been reported in Malaysia (Knight et al., 1992), Myanmar (Han and Hlaing, 1989), Bangladesh (Haque et al., 1999), and Indonesia (Gasem et al., 2001). In addition, in subjects who have pervious history of infection and have taken anti-amoebic chemotherapy have 1.4 and 1.5 fold increase risk of *E. histolytica* infection suggesting the possibility of drug resistant strains among North Eastern population. However, further studies are needed in this respect particularly focusing on the metronidazole.
sensitivity of natural and clinical isolates and expression of enzymes implicated in metronidazole resistance. Since human-to-human transmission is a common mode, screening and successive anti-amoebic treatment of the infected family persons based on one affected member would appear to be justified. Identification of *E. histolytica* strains in a clinical setting and their association with virulence patterns need to be justified.

We observed that there is a significant higher prevalence of *E. histolytica/E. dispar/E. moshkovskii* infection among asymptomatic non HIV subjects. It is well accepted that 90% of *E. histolytica/E. dispar/E. moshkovskii* infected cases are asymptomatic (Stanley, 2003). The possibility of harbouring the non-pathogenic species cannot be ruled out. Moreover, it is now well documented that *E. dispar* infection is much more prevalent than *E. histolytica* worldwide (Ramos et al., 2005; Leiva et al., 2005). In Agboville town, PCR analysis of microscopically positive samples demonstrated the ratio of *E. histolytica* to *E. dispar* of 1:46 (Heckendorn et al., 2002). Infections with *E. moshkovskii* have also been reported in Bangladesh, India, Iran, Turkey, Australia, Tanzania and Malyasia and usually they are not associated with disease (Haque et al., 1998; Parija and Khairnar, 2005; Solaymani-Mohammadi et al., 2006; Tanyuksel et al., 2007; Fotedar et al., 2008; Beck et al., 2008; Anuar et al., 2012b). However, in our study, 7 individuals mono-infected with *E. moshkovskii* were symptomatic. Study in India and Malaysia also found association of *E. moshkovskii* infection with dysentery (Parija and Khairnar, 2005; Anuar et al., 2012b). However, more studies are necessary to validate the role of *E. moshkovskii* in gastroenteritis disorders and its virulence. While concerning seasonal prevalence likewise *E. histolytica* prevalence, the E complex prevalence also follows the same pattern with highest prevalence in the monsoon period further highlighted the high rate of wide spreading and faecal–oral contamination during the monsoon season particularly in flood plain area like Assam.

Association studies have shown that being a consumer of raw vegetables presents a 1.6 fold higher risk of acquiring *Entamoeba* complex infection. This finding was in line with previous studies done in Brazil and Iran highlighting the potential of unwashed raw vegetables in the transmission of *Entamoeba* (Benetton et al., 2005; Shahnazi and Jafari-Sabet, 2010). The present study also highlights that infection
history in the family members were 3.0 times more likely to be infected with *E. histolytica*/*E. dispar*/*E. moshkovskii*. Ostan et al. (2007) also claimed that person-to-person transmission is the most important determinant of infection. A study conducted among Mexican population found that 40% of individuals in contacts of *E. histolytica*/*E. dispar* carriers were also infected (Ruiz-Palacios et al., 1992). In El Salvador, contacts of individuals with asymptomatic cyst carriers, with amoebic dysentery or Amoebic liver abscess patients were found as important predictors of higher parasite infection rates in compared with their counterparts (Spencer et al., 1981). Similarly, Shahrul Anuar et al. (2012) reported those living with family members already infected with *E. histolytica*/*E. dispar*/*E. moshkovskii* were at 2.62, 4.92 and 12.32 fold higher risk of being infected among three Orang Asli Ethnic Groups in Malaysia.

Our analysis also confirmed that poor living condition, unhygienic toilet facility, not washing hands always before taking food are important predictors of *Entamoeba* complex infection. Previous study conducted in Italy and Yemen also showed that individuals who do not have the habit of hand washing before eating are at higher risk (two fold) of *E. histolytica*/*E. dispar* infection (Seppo et al., 2005; Naelah et al., 2011). In addition, interestingly we observed *E. histolytica*/*E. dispar*/*E. moshkovskii* infection was significantly higher among individuals with close contacts with domestic animal which is around three fold higher compare to participants who were not in close contact with domestic animals. This present study once again highlights the significant association of parasite infection and close contact with domestic animals, especially dogs and cats. Analogous results were also observed in other endemic areas in Yemen (Naelah et al., 2011), Vietnam (Pham Duc et al., 2011) and Malaysia (Anuar et al., 2012a). Wittnich (1976) has reported case of *E. histolytica* infection in a German shepherd dog. Recently, *E. hartmanni, E. coli* and *E. dispar* were isolated from captive non-human primates housed in the zoological garden of Rome highlighting the risk of zoonotic transmission of this parasite for animal caretakers and visitors (Berrilli et al., 2011). To understand the actual dynamics of transmission in North Eastern population particular those are in close contact with domestic animal genotyping of *E. histolytica*/*E. dispar*/*E. moshkovskii* from humans and animals are highly recommended.